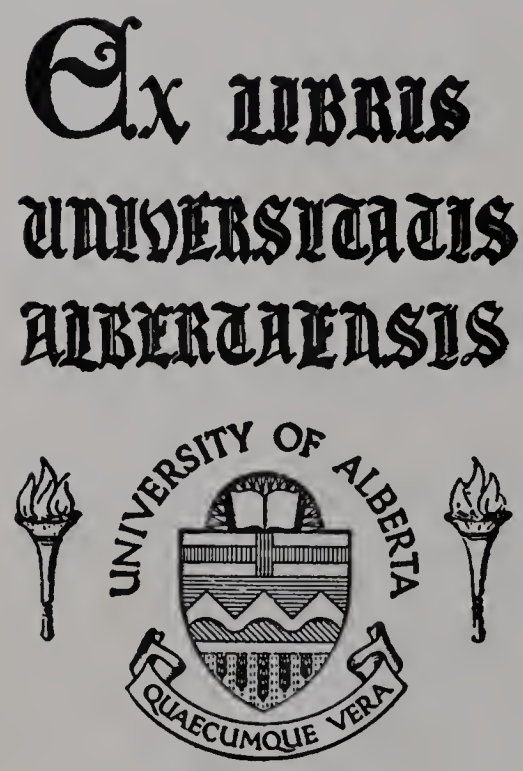

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ACETATE METABOLISM IN A COLIFORM ISOLATED FROM BOVINE RUMEN FLUID

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Acetate metabolism in a coliform isolated from bovine rumen fluid" submitted by Cuddalore Rajagopal Krishnamurti, B.V.Sc., M.V.Sc., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Bacterial counts on the rumen fluid of 2 cows fed alfalfa hay revealed the presence of coliform organisms in a concentration in excess of 2% of the total microbial population. When grain was included in the ration of one of the cows, the viable coliform count was of the order of 2.3×10^9 per ml as compared to a maximum of 4×10^8 per ml when hay alone was fed. However, total microscopic counts were also higher when grain was fed so that the ratio of viable coliform counts to total microscopic counts was not changed. Diurnal variations were observed in viable coliform counts; the numbers increased immediately after feeding and fell off gradually to the pre-feeding level after 10 hours.

A coliform organism was isolated from a 10^{-8} dilution of rumen fluid from a cow fed alfalfa hay. Based on morphological, cultural, physiological and biochemical characteristics, the organism was identified as Escherichia coli Type I and designated E. coli 64.

Under aerobic conditions, E. coli 64 entered the exponential phase of growth after 4 hours of incubation with glucose or lactate as substrates, and after 5-6 hours when incubated with acetate or glycollate. Under anaerobic conditions, slow growth occurred, when the organism was incubated with glucose or lactate, but no growth was observed with acetate or glycollate as substrates.

When cells of E. coli 64 were harvested in their exponential phase of growth in an acetate medium and incubated aerobically with sodium acetate-2-C^{14}, about 33% of the label appeared in CO_2. Of the radioactivity in the cells, 72% was recovered in the protein hydrolysate, 8% in the nucleic acid, 6% in the lipid and 14% in the ethanol soluble

fractions. The radioactivity in the protein hydrolysate of cells incubated with sodium acetate-2-C^{14} was approximately 20 times that in the hydrolysate of cells incubated with $C^{14}O_2$ as the carbon source. This showed that CO_2 fixation reactions alone could not provide for regeneration of all the C_4 dicarboxylic acids drained from the Krebs cycle for synthetic purposes when cells were incubated with acetate as the sole source of carbon. The possibility that the glyoxylate cycle might be present in E. coli 64 was therefore examined.

Cell-free extracts of the organism were prepared by centrifugation after sonic disintegration of the cells in a buffer solution. By spectrophotometric methods, it was demonstrated that extracts of cells grown on acetate contained acetate kinase and phosphate acetyltransferase, which are common to the Krebs and the glyoxylate cycles, plus, as demonstrated by spectrophotometric and isotopic methods, isocitrate lyase and malate synthase which are characteristic of the glyoxylate cycle. The enzymes of the glyoxylate cycle could not be demonstrated in cell-free extracts of E. coli 64 grown on glucose under either aerobic or anaerobic conditions.

Possible functions that E. coli 64 may have in maintenance of anaerobiosis in the rumen, utilization of acetate through the glyoxylate pathway and utilization of lactate are discussed.

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INTRODUCTION

The importance of rumen microflora in the digestion of cellulose in the rumen was recognized as early as 1883 by Tappeiner. The bulk of the work published on rumen bacteria has been devoted to the metabolism and nutritional requirements of obligate anaerobic microorganisms concerned with cellulose digestion and volatile fatty acid production. Although obligate anaerobes constitute the major part of the rumen microbial population, facultative anaerobes are normally present in appreciable numbers. Inasmuch as rumen facultative anaerobes have not been thoroughly studied, comparatively little is known regarding their metabolic pathways. Furthermore, the possibility cannot be excluded that they may make significant, or even essential, contributions to normal rumen fermentation processes.

Coliforms are among the facultative anaerobes normally present in the rumen. In this investigation, a coliform which was present in a high dilution of rumen fluid from a cow fed alfalfa hay was isolated and metabolic pathways by which the isolate could use acetate were studied in detail.

REVIEW OF LITERATURE

Facultative anaerobes in the rumen

Most of the pure culture studies reported on rumen bacteria have been devoted to obligate anaerobic organisms concerned in cellulose digestion and volatile fatty acid (VFA) production. In order to distinguish true rumen bacteria from 'casual' organisms ingested along with feed and water, Gall and Huhtanen (1951) proposed five criteria of which strict anaerobic nature is one. In a recent review, Hungate et al. (1964) use the term 'euryoxic' to indicate an ability to live under a wide range of oxygen tension and state that euryoxic bacteria are less abundant in the rumen and of uncertain significance. However, it has been suggested that "there can be no a priori reason why facultative anaerobic bacteria should not be present and be active in large numbers in the rumen," and that one factor in favor of this argument is that rations fed to ruminants contain not only cellulose but other carbohydrate constituents such as sucrose and grass levans which are not fermented by strictly anaerobic cellulolytic bacteria (Heald et al., 1953).

Among the facultative anaerobes, the most widely studied organism is Streptococcus bovis. Its ability to digest starch, capacity to produce dextran from sucrose and reported involvement in certain metabolic disorders, such as acute acid indigestion and bloat, have focussed attention on this organism (Hungate et al., 1952; Bailey and Oxford, 1958a, b). Facultative anaerobes have also been reported to be associated with proteolysis in the rumen (Appleby, 1955; Hunt and Moore, 1958).

Only meagre information is available regarding the incidence and metabolism of rumen strains of coliform organisms. During a study of pentosan fermentation in the rumen, Heald (1952a) isolated 17 strains of

Escherichia coli from xylose fermenting cultures. Lactic acid, acetoin and 2,3-butanediol were not formed from xylose and glucuronic acid whereas they were formed from glucose and cellobiose, indicating that with the former substrates either pyruvic acid was not formed, or that the mechanisms involved in the subsequent formation of both lactic acid and acetoin were not operative. Heald (1952b) further observed that coliform bacteria growing on xylose formed adaptive enzymes for the fermentation of glucuronic and galacturonic acids and l-arabinose. Hobson and Purdom (1961), however, reported that facultative anaerobic bacteria are not active in the ruminal fermentation of xylans.

Heald et al. (1953) estimated that crude wet rumen contents of a sheep fed hay contained 10^8 viable facultative anaerobic Gram-positive cocci and 10^6 Gram-negative rods, including coliform bacteria, per gram. Mann et al. (1954) found that the number of coliforms was of the order of 10^4-10^5 per gram of wet rumen contents from sheep. Most of the 150 isolates of coliforms were typical intestinal E. coli. A few strains resembled Aerobacter cloacae but A. aerogenes was not encountered.

The rumen coliform population of 12 calves of ages 10-105 days was studied by Mackay and Oxford (1954). The number was never greater than 10^6 per gram and consisted mostly of E. coli and intermediate types. Bryant et al. (1958) observed that lactate fermenting bacteria were present in high numbers at 1-3 weeks of age but declined to numbers similar to those of the adult at 9-13 weeks. Coliforms were highest in numbers when calves were 1-3 weeks of age; even at 13 weeks these counts were higher than those of the adult. The study of Ziolecki and Briggs (1961) on the microflora of the rumen of calves during the first week of life showed that the coli-aerogenes count (10^7-10^8 per gram wet weight) was higher

than in the adult rumen but dropped to its normal level during the succeeding 2 weeks. Most of the coli-aerogenes organisms were E. coli fecal Type I.

Dohner and Cardon (1954) studied the morphological, cultural and physiological characteristics of two strains of E. coli isolated from the bovine rumen. They considered these two organisms as common inhabitants of the rumen since they were isolated from each of the five enrichment cultures started. A quantitative bacteriological study of rumen contents from 3 fistulated cows by Wilson and Briggs (1955) revealed that Gram-negative rods of the coli-aerogenes type were rarely present beyond the 10^{-6} and 10^{-7} dilutions. Davey and Briggs (1959) observed high similarity in the coliform counts between complementary samples of cud and rumen contents of cows. Coliform counts were of the order of 10^{7}-10^{8} per ml of fluid; four types were differentiated, viz., E. coli Type I, Intermediate Type I, Intermediate Type II and A. aerogenes Type I.

Utilization of acetate by rumen microorganisms

The chief end products of fermentation in the rumen are the lower volatile fatty acids, methane and CO_2. Volatile fatty acids accumulate in the rumen because they are not susceptible to rapid further breakdown by the mixed microbial population (Warner, 1964). There is, however, good evidence that at least limited amounts of acetic and other volatile fatty acids are utilized by rumen organisms.

Bryant and Doetsch (1955) studied the nutritional requirements of Bacteroides succinogenes, an important rumen cellulolytic anaerobe, and found that it required a combination of n-valeric or n-caproic acid, both of which are considered to be formed from acetate, plus a branched-chain fatty acid - isobutyric, isovaleric or DL- α -methyl n-butyric.

The nutritional requirements of other cellulolytic organisms, Ruminococcus flavefaciens and R. albus, were studied by Allison et al. (1958). They found that deletion of acetate from the medium resulted in a marked increase in the incubation time needed to reach maximum growth. Wegner and Foster (1960) reported similar fatty acid requirements for many of their rumen isolates, and Bryant and Robinson (1962), working with 89 strains of culturable ruminal bacteria, provided further evidence that acetate as well as 4- and 5-C fatty acids are important nutrients for many species of rumen bacteria.

Hoover et al. (1963) investigated the relative rates at which VFA and glucose were utilized by rumen bacteria and compared them as carbon sources for in vitro protein synthesis. From the decline in the specific activities of labeled substrates they estimated that the average rates of utilization for acetic, propionic, butyric and valeric acids were 131, 18.2, 13.2 and 0.6 mg/liter/hour respectively. The rate of glucose utilization was proportional to the amount added and was 556 mg/liter/hour at a level of 10 mg per ml. It was also observed that, of the activity that disappeared from labeled acetate, propionate, butyrate, valerate and glucose, 2.5, 2.1, 1.6, 2.6 and 2.9% respectively was incorporated into proteins of the bacteria.

Keeney et al. (1962) found that rumen bacteria contained several branched- and straight-chain odd number carbon fatty acids that, in minor quantities, are characteristic of milk and ruminant tissue. Katz and Keeney (1964) showed that the carbon skeletons of the odd number carbon fatty aldehydes in rumen bacteria are also similar to those in the ruminant lipids. It is generally assumed that the condensation of acetate and propionate units is responsible for the formation of these higher fatty acids with an odd number of carbon atoms, and that the condensation of

acetic with isobutyric, isovaleric or 2-methylbutyric acid is responsible for the formation of branched-chain higher fatty acids.

Acetate metabolism in bacteria and elucidation of the glyoxylate cycle

The establishment of the existence of the Krebs cycle in animal tissues by Krebs (1950) prompted microbiologists to seek for its existence in bacteria. Ajl and Kamen (1951) studied the oxidation of acetate by E. coli strain E26 and found that, when incubated with C^{14} labeled acetate and unlabeled α-ketoglutarate, no isotope was incorporated into the latter compound. They therefore concluded that Krebs cycle was not active in the metabolism of acetate. However, study of metabolic pathways by carrier techniques may, as has been indicated by Swim and Krampitz (1954a), be misleading because of non-equilibrium between the carrier and metabolically formed intermediates. They incubated E. coli cells with acetate-2-C^{14} in the absence of an added carrier and found that the cycle intermediates were in isotopic equilibrium with each other and in approximate equilibrium with residual acetate, thus confirming the presence of the Krebs cycle. Later (1954b) they furnished evidence for the quantitative significance of the Krebs cycle by studying the anaerobic metabolism of acetate by E. coli in the presence of fumarate as an oxidant which prevented recycling of the succinate formed. By mass spectrographic analysis they observed that all the succinate synthesized from acetate-2-C^{13} under these conditions was singly labeled in the methylene position; this indicated that succinate was formed through the Krebs cycle and that a Thunberg type condensation did not occur.

The above metabolic pattern observed with non-proliferating cell suspensions represents the catabolism of acetate for energy but not the mode of utilization for growth. Cutinelli et al. (1951) studied the metabolism of labeled acetate in E. coli and, from the labeling patterns

of the amino acids formed, concluded that acetate was incorporated into protein via the Krebs cycle. Roberts et al. (1955) provided conclusive evidence that acetate and intermediates from the Krebs cycle are used for synthetic processes. Thus, during growth on acetate as the sole source of carbon, the continuous draining of intermediates from the Krebs cycle for synthetic purposes would ultimately cause the cycle to stop, if the intermediates were not replenished by some means. Investigations which culminated in the elucidation of pathways by which replenishment could be accomplished are summarized below.

Evidence for the existence, in bacteria, of a possible deviation from the conventional Krebs cycle was presented by Campbell et al. (1953). They incubated cell-free extracts of Pseudomonas aeruginosa 9027 with citrate, or cis-aconitate and found that glyoxylic acid was formed but not α-ketoglutarate. They suggested that a cyclic mechanism similar to the Krebs cycle, with the exception of the steps from cis-aconitate to succinate, was present in this organism. In the experiments of Campbell et al., isocitrate was not dissimilated, but Saz (1954) reported that it too is attacked by cell-free extracts of both Ps. aeruginosa and Ps. fludrescens. Using partially purified cell-free extracts of Ps. aeruginosa, Smith and Gunsalus (1954) showed the stoichiometry of the reactions to be: isocitrate $\xrightarrow{\text{isocitritase}}$ glyoxylic acid + succinic acid. They could not demonstrate reversibility for isocitritase with either crude or fractionated preparations. A similar d-isocitric lyase system which catalyzed the formation of glyoxylic and succinic acids from d-isocitric acid was demonstrated in cell-free extracts of Penicillium chrysogenum by Olson (1954). Smith and Gunsalus (1955) showed that the isocitritase content of several species of bacteria varied with growth conditions; for example, isocitritase could be demonstrated in E. coli Crooks strain only if the

cells were grown aerobically with acetate as the sole source of energy.

Wong and Ajl (1955) demonstrated the presence of isocitritase in E. coli strain E 26 grown on acetate. They originally assumed that the glyoxylate formed by the cleavage of isocitrate was oxidized to CO_2 and water, thus providing an alternate route for the complete combustion of acetate; but later (1956) they demonstrated that the organism produced malate synthetase which converted glyoxylate and acetate to malate — acetyl CoA + glyoxylate $\xrightarrow{\text{malate synthetase}}$ malate + CoA. In 1957 Wong and Ajl suggested that the significance of malate synthetase is that it enables bacteria growing on C_2 compounds to form new C_4 compounds which enter the Krebs cycle and permit it to continue to serve as a mechanism for production of both energy and cell constituents. But they could not establish with certainty whether the conversion of acetate to glyoxylate proceeded through a C_6 intermediate or by a direct conversion through glycollate.

In the light of these experiments, Kornberg (1958) investigated the growth of Ps. fluorescens KBI on acetate as the sole source of carbon. He incubated cells with C^{14} labeled acetate and observed that over 70% of the total isotope was present in the C_4 dicarboxylic acids after a very few seconds, but after establishment of an isotopic steady state, citrate and glutamate had higher activity than the dicarboxylic acids. The results suggested that acetate entered the Krebs cycle at two sites, to form citrate from one and C_4 compounds from the other.

The possibility that CO_2 fixing mechanisms may account for the regeneration of the C_4 acids drained from the Krebs cycle during synthetic processes was investigated by Kornberg (1956) and Kornberg and Quayle (1958). They concluded that the known CO_2 fixation reactions were quantitatively inadequate to account for the growth of Ps. fluorescens KBI on acetate as the sole source of carbon.

At the same time Kornberg and Quayle degraded aspartic acid and alanine from cells grown on acetate-2-C^{14} in the absence of CO_2 and found that the percentages of labeling in the carbon atoms were,

35	COOH	99	CH_3
100	CH_2	100	$CHNH_2$
100	$CHNH_2$	41	COOH
35	COOH		
aspartic acid		alanine	

Had the Krebs cycle been the only mechanism in operation (cf. Fig. 1), the percentages of labeling would have been,

50	COOH	100	CH_3
100	CH_2	100	$CHNH_2$
100	$CHNH_2$	50	COOH
50	COOH		
aspartic acid		alanine	

The fact that appreciably less labeling of carboxyl carbons occurred than would have been the case had the Krebs cycle been the only pathway for incorporation of acetate into the amino acids, suggested the existence of another pathway for incorporation of the carboxyl group of acetate. The demonstration by Kornberg and Madsen (1957, 1958) of the presence of both isocitritase and malate synthetase in the extracts, led them to unify the fragmentary reports on these enzymes and offer the 'glyoxylate by-pass' of the Krebs cycle as an auxiliary pathway for the formation of C_4 dicarboxylic acids,

$$\text{isocitrate} \xrightarrow{\text{isocitritase}} \text{succinate} + \text{glyoxylate}$$

$$\text{glyoxylate} + \text{acetyl CoA} \xrightarrow{\text{malate synthetase}} \text{malate} + \text{CoA}.$$

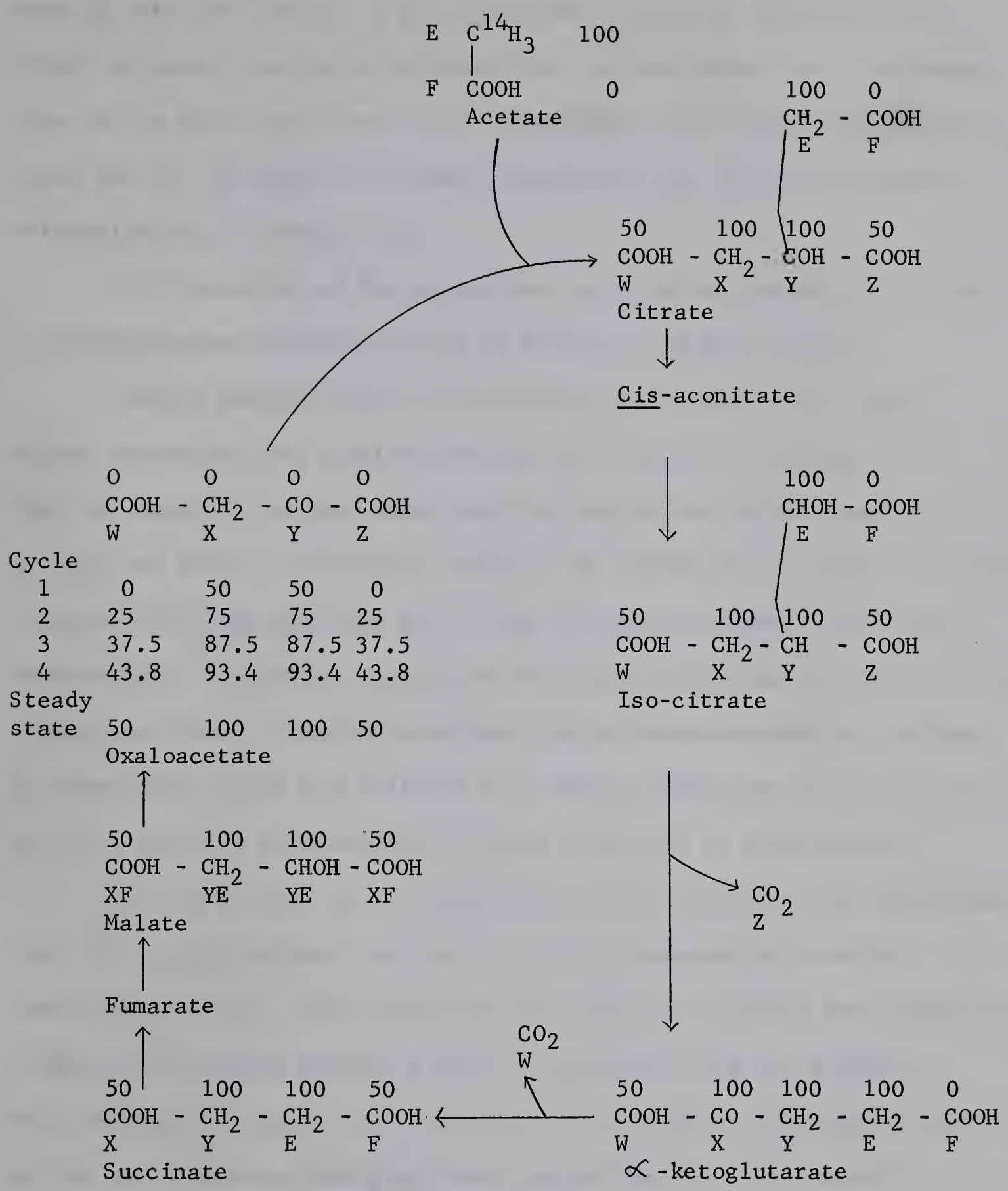


Fig. 1. Abridged Krebs cycle showing the percentage labeling of carbon atoms in intermediates formed from acetate-2-C^{14} under steady state conditions. Carbon atoms from acetate are marked as E and F and those from oxaloacetate as W, X, Y and Z.

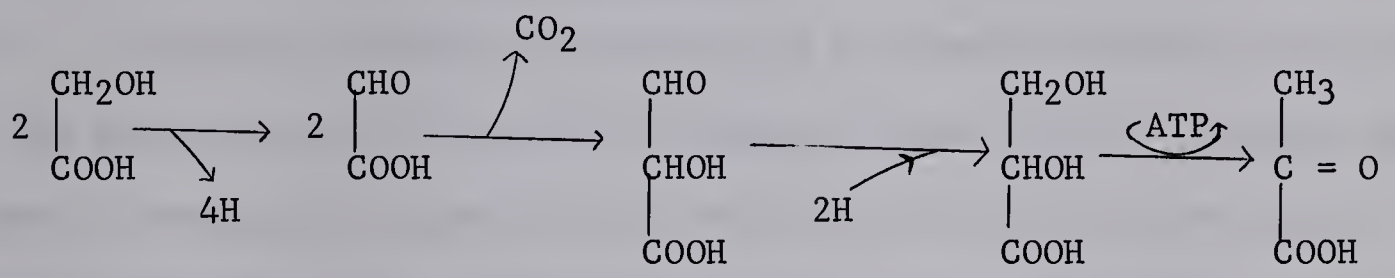
Working with two strains of E. coli grown on acetate, Kornberg et al. (1960) obtained conclusive evidence that the net formation of intermediates of the Krebs cycle could be accomplished only through the glyoxylate cycle and not through the Thunberg condensation or through successive carboxylations of acetic acid.

The operation of the glyoxylate cycle in the conversion of fat to carbohydrates was demonstrated by Kornberg and Krebs (1957).

Reeves and Ajl (1960, 1962) demonstrated that, in E. coli, malate synthetase and isocitritase are not present in resting cells; they are adaptive enzymes which must be synthesized before growth of E. coli can occur in an acetate medium; the length of the lag period varies inversely with the specific activities of the two enzymes. They also observed that the specific activity of malate synthetase was consistently higher than that of isocitritase when the cells were grown on a variety of substrates, which may indicate that malate synthetase is constitutive. In cells grown on glucose, isocitritase could not be demonstrated.

During a study on the regulation of the enzymes of the glyoxylate cycle in E. coli mutants lacking a $C_3 + CO_2$ condensation reaction, Vanderwinkel et al. (1963) observed that growth on acetate was completely inhibited by glucose whereas growth on glycollate was not affected. This led them to suggest the existence of two malate synthases involved in the use of acetate and glycollate respectively. Very recently Falmagne et al. (1965) confirmed this hypothesis by showing that purified preparations of malate synthases isolated from the extracts of cells grown on acetate or glycollate had different physical properties and behaved differently towards inhibitors.

It is generally assumed that the glyoxylate cycle is not present in organisms capable of growing on C_2 compounds more oxidized than acetate. In such cases the 'glycerate pathway' has been postulated to account for the net formation of Krebs cycle intermediates (Kornberg, 1961). According to this scheme, 2 moles of C_2 compound, glycollate for example, are converted to 1 mole of CO_2 and 1 mole of pyruvate as follows,



The pyruvic acid is either carboxylated to oxaloacetic acid or is oxidized to acetyl CoA, which condenses with a third molecule of glyoxylic acid to yield malic acid (Kornberg and Sadler, 1961).

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

MICROBIOLOGICAL STUDIES

Experiment 1
Incidence of coliform organisms in the rumen

Introduction

Coliform organisms are recognized as almost universal inhabitants of the gastro-intestinal tracts of mammals (Smith, 1965). Because the rumen is essentially anaerobic and because cellulolysis is of major importance in the rumen, obligate anaerobic rumen cellulolytic organisms have received much more study than have the facultative anaerobes that are known to be present in the rumen in appreciable numbers under most conditions. The facultative anaerobe that has received most consideration is Streptococcus bovis. Under normal conditions of feeding and management coliforms constitute only a small fraction of the total rumen microbial population, but this does not preclude the possibility that they may play a significant role in the total complex of events concerned in either normal or abnormal rumen microbial metabolism. Not only do the substrates available to rumen and intestinal coliforms differ in that volatile fatty acids rather than hexoses constitute the major energy forms available from carbohydrate digestion in the rumen, but the pH and the redox-potential in the rumen are lower than in the intestine. Because the relative abundance of coliforms might be expected to be related to their possible contribution to metabolic processes in the rumen and because their incidence might be expected to vary according to the ration available to the host, the object of the first experiment was to study the incidence of coliforms in the rumen under conditions of hay versus hay-grain feeding.

Experimental

Experimental animals

Two mature Jersey cows with permanent rumen fistulae served as the source of rumen liquor. They were maintained at the University of Alberta livestock farm and fed 15 lb. of good alfalfa hay at 7:00 AM and 6:00 PM. They had been fed this ration with free access to salt and bonemeal in mineral boxes for over a year before the commencement of the present experiments. Water was available ad lib. from an automatic water bowl. One of the cows (Lyssa) calved on July 8, 1965. After parturition this cow developed metritis and was no longer used for the collection of rumen fluid. The condition of the other cow (Calla) was good throughout.

Rumen fluid was obtained by manually removing through the fistula a total of about 4 lb. of rumen contents from different parts of the rumen. This was squeezed through four layers of cheesecloth in a squeeze press. The rumen liquor so expressed was collected in sterile containers at ambient temperature and taken to the laboratory as quickly as possible for bacteriological examination. With the exception of those drawn to obtain data on diurnal variation in viable coliform counts, samples were taken at approximately 10:00 AM.

To study the effect of the inclusion of grain in the ration on the incidence of coliform organisms in the rumen, the ration of one of the cows (Calla) was changed to a 50-50 mixture of alfalfa hay and equal parts of oats and barley. After she had been on this ration for 15 days, rumen liquor was collected for coliform counts.

Microbiological methods

Viable counts of coliform organisms in the rumen fluid were made by standard surface and pour plate methods using MacConkey agar. Since this medium suppresses the growth of Gram-positive organisms and contains

a fermentable carbohydrate, lactose, which, on fermentation, acts on the incorporated indicator to impart a pink color to colonies of bacteria which ferment lactose, it serves as a differential as well as a selective medium for the study of coliforms.

Annison and Lewis (1959) suggested that the significance of the differential viable count can be assessed only in relation to the total viable count. Because a single culture medium may not be able to support the growth of all the physiologically divergent organisms and because the total microscopic count is less variable than the total viable count, it was hypothesized that the ratio of the viable count to the total microscopic count may be a better yardstick of the numerical importance of an organism in the rumen than the viable count alone. Accordingly counts for total number of bacteria in samples were done in a haemocytometer after suitable dilution. The details of these procedures are given in Appendix p. 1.

Results

By the surface plate method, the average viable coliform count, for three plates per sample of rumen fluid taken from Lyssa while she was being fed hay, varied from 10^6 to 10^8 organisms per milliliter. For Calla, the viable counts varied from 10^7 to 10^8 for the December, January and March samples, but for the July sample the count fell to the order of 10^4 per ml of rumen fluid (Table 1).

Less variation was observed in total microscopic counts; for both cows the range was of the order of 10^9-10^{10} organisms per milliliter. For Lyssa, the proportion of viable coliforms to total organisms varied from 0.5 to 6.7%, whereas for Calla the range was from 0.003 to 4%. If the unexplainable divergent July results for Calla were arbitrarily excluded, the average proportion of coliforms in the total microbial population

would be 3% versus an average of 2.7% for all eight observations. Despite their high variability, the results suggest that coliforms are likely to constitute an appreciable fraction of the total rumen population in cattle maintained on hay rations. A reduction in the viable coliform count to the extent of approximately 10^3 organisms per ml was observed with pour plates as compared to surface spread plates.

Unfortunately, circumstances permitted only one test on rumen fluid from one cow (Calla) under conditions of feeding hay plus grain. Taking into account the high variability in the results obtained when the ration consisted of hay alone, the results (Table 2) are, of course, inconclusive; at most they simply suggest that the coliform and total microscopic counts were higher when hay plus grain rather than hay alone was fed, but that the proportion of coliforms to the total number of bacteria remained approximately the same.

Study of the diurnal variations in the numbers of coliforms in the rumen fluid was limited to one test with samples taken from both cows on March 3, 1965. Three hours after feeding, the viable count of coliforms was 6 to 25 times, and at 7 hours, 2 to 10 times higher than in the pre-feeding period (Table 3).

Discussion

The viable coliform counts obtained in this study are slightly higher than those reported by Wilson and Briggs (1955) who used a 'reinforced clostridial medium' and found that Gram-negative rods of the coli-aerogenes type were rarely present beyond the 10^{-6}-10^{-7} dilutions of rumen fluid from three adult cows fed grass, hay or concentrates. For the enumeration of coliform organisms, the use in this study of the more selective MacConkey medium yielded slightly higher counts than the medium used by Wilson and Briggs (1955).

Table 1. Viable coliform and total microscopic counts in rumen fluid from cows fed alfalfa hay

Date	Cow: Lyssa			Cow: Calla		
	Avg* viable coliform count per ml	Total microscopic count per ml	% viable coliform of total count	Avg* viable coliform count per ml	Total microscopic count per ml	% viable coliform of total count
Surface plates:						
Dec. 1/64	3×10^8	2×10^{10}	1.5	4×10^8	10^{10}	4.0
Jan. 15/65	2×10^8	3×10^9	6.7	4×10^8	10^{10}	4.0
Mar. 3/65	5×10^6	10^9	0.5	1.3×10^7	10^9	1.3
July 1/65	6.6×10^7	2×10^9	3.3	3.3×10^4	10^9	0.003
Avg	1.43×10^8	6.5×10^9	3.0	1.38×10^8	5.5×10^9	2.3
	Maximum viable coliform count per ml					
Pour plates:						
Dec. 1/64	8×10^6			5×10^5		
Jan. 15/65	6×10^5			5×10^5		
Mar. 3/65	3×10^3			2×10^4		
July 1/65	6×10^5			4×10^3		
Avg	2.3×10^5			2.3×10^5		

*3 plates per sample

Table 2. Viable coliform and total microscopic counts in rumen fluid from Calla after she had been fed a ration containing 50% grain for 15 days

Date	Average* viable coliform count per ml	Total microscopic count per ml	% viable coliform of total count
Sept. 27/65	2.3×10^9	10^{11}	2.3

*3 plates per sample

Table 3. Diurnal variations in viable coliform counts

Time	Hours after feeding	Cow: Lyssa — Viable coliform count per ml	Cow: Calla — Viable coliform count per ml
Mar. 3/65			
6:00 AM	12	200,000	2,000,000
10:00 AM	3	5,000,000	13,000,000
2:00 PM	7	2,000,000	4,000,000
5:00 PM	10	300,000	1,000,000

Using the pour plate method with violet red-bile agar, Bryant et al. (1958) observed only 2600 coliforms per ml of rumen fluid from a mature Holstein cow fed 10.1 lb. of alfalfa hay and 5.1 lb. of grain per day versus 230,000 coliforms per ml observed in the present study. A number of factors such as differences in counting techniques, rations, and perhaps particularly sampling errors associated with uncontrolled dilution effects of water ingested, might be expected to contribute materially to variability in results obtained by different investigators as well as in the present study. Both the cows in this study were allowed drinking water ad lib. from an automatic water bowl, so that depending on when and how much water they drank, the consistency of the rumen liquor varied. In the reports of Wilson and Briggs (1955) and Bryant et al. (1958), no mention is made as to whether access to water was controlled.

The consistency of the rumen contents used for cultural investigation might have affected the counts. Baker (1943) recognized two types of rumen organisms, the 'fixed type' which includes the cellulose digesting bacteria found invariably attached to the fibers and the 'free forms', including coliforms, which attack soluble substrates. Wilson and Briggs (1955) used mixed solid and liquid rumen contents for their study whereas only the liquid portion was used here. This probably explains the higher coliform counts observed in this study.

The fact that less variation was observed in the ratio of viable coliform counts to total microscopic counts than in the viable counts alone, under conditions of feeding hay or hay plus grain, supports the hypothesis that this ratio may be useful to assess the numerical importance of any organism in the rumen.

Hobson (1963) has suggested that the rumen may be likened to a continuous culture of mixed rumen microorganisms with bursts of activity

of one or more types of organisms depending on the inflow of feed. Raguse and Smith (1965) have reported that there was a total available carbohydrate content of about 10% in air dried alfalfa herbage. Presumably increased availability of soluble carbohydrates contributed to the increase in number of coliforms immediately after feeding in the present experiment. Also, an increase in the amount of oxygen in the rumen might be expected to accompany the ingestion of food, and this would tend to stimulate the growth of facultative anaerobes such as the coliforms. The reduction in oxygen tension associated with their rapid growth would, in turn, provide improved conditions for the growth of obligate cellulolytic anaerobes.

Among the factors contributing to the gradual fall in coliform numbers after the initial increase, may be decreased readily available substrate and predatory activity of the protozoa. Using C^{14} labeled *E. coli* and other bacteria, Coleman (1964) provided evidence for ingestion of bacteria by the ciliate protozoa, *Entodinium caudatum*. This, coupled with the observation that there is an inverse relation between the diurnal variation of protozoa (Purser and Moir, 1959) and that of the coliform organisms, suggests that the latter may be ingested by the protozoa.

Summary

1. Numbers of coliform organisms in samples of rumen fluid from cows fed alfalfa hay were counted using MacConkey agar. Numbers varied from 10^6-10^8 organisms/ml of rumen fluid. Coliform organisms constituted a minimum of 0.003%, a maximum of 6.7% and an average of 2.7% of the total microscopic count.
2. When grain was included in the ration of one of the cows, there was a rise in the viable coliform count to 2.3×10^9/ml versus a maximum of 4×10^8/ml on hay alone.

3. No indication was obtained that feeding hay alone versus feeding equal parts of hay and grain affected the proportion of viable coliforms to total rumen microorganisms.
4. A diurnal variation was observed in the viable count of coliform organisms, the number increasing shortly after feeding and falling off gradually to the pre-feeding level after 10 hours.

Experiment 2
Isolation and characterization of a rumen coliform organism

Introduction

Many workers have isolated coliform organisms from the rumen and studied their general characteristics. Heald (1952a) in particular has shown that rumen coliforms are actively associated with the fermentation of soluble carbohydrates. Inasmuch as there are many advantages to the use of pure, rather than mixed cultures in experiments designed to study metabolic processes in bacteria, it was decided to isolate a single coliform organism and employ pure cultures of it in the experiments planned. The isolation was made from a high dilution of rumen liquor in the hope that an organism which normally occurs in high numbers in the rumen might thereby be obtained. The method of isolation and the morphological, cultural, biochemical and physiological characteristics of the coliform isolate are described below.

Experimental

Isolation

Several MacConkey agar plates were inoculated with a dilution of 10^{-8} of rumen fluid from a cow (Lyssa) fed alfalfa hay. One of the pink colonies was picked and subcultured in nutrient broth. Gram stained smears were employed for microscopical examination of the culture. Motility was observed by a hanging drop method. Stock cultures of the organism were maintained on nutrient agar slants. Subcultures on fresh media were made at 15-day intervals.

Biochemical reactions and fermentation tests

These were done by standard methods -- Cruickshank (1960), Manual of Microbiological Methods (1957). The details are given in Appendix p. 2.

Growth on different energy substrates

To test the growth rates of the organism on different energy sources, it was grown in 125 ml Erlenmeyer flasks in the following basal medium, supplemented with 0.5% of the appropriate energy source:

	g
$(NH_4)_2SO_4$	0.09
NaCl	0.09
$MgSO_4.7H_2O$	0.01
Yeast extract (Difco)	0.02
Phosphate buffer (0.1M, pH 7.0)	100 ml

Inoculation from a saline suspension of the organism was made into each medium using a standard loop; the flasks were incubated on a horizontal shaker at 37 C. Optical densities at various intervals of incubation were determined in a Bausch and Lomb Spectronic 20 colorimeter at 625 mμ, using an uninoculated medium as the blank.

Results

Morphological

The isolated coliform organism is a short rod with parallel sides and rounded ends (Fig. 2); the average length is 2 μ and the width 0.4 μ; the rods usually appear in pairs; cocco-bacillary forms are also seen; the organism is Gram-negative and sluggishly motile.

Cultural

The isolate is aerobic and facultatively anaerobic. It grows aerobically and anaerobically on glucose or lactate as single sources of carbon; it grows at a slower rate on acetate or glycollate under aerobic conditions, but no growth was observed under anaerobic conditions with acetate or glycollate. Paper chromatographic analysis of the fermentation products after incubation with lactate anaerobically revealed formic acid as the major product plus traces of acetic acid. On nutrient agar, moderately large, thick and greyish-white, moist, circular, smooth,

low-convex, opaque colonies develop in 24 hr at 37 C. On MacConkey agar the colonies are similar to those found on nutrient agar except that they are pink. In broth there is abundant growth with uniform turbidity. After 24-72 hr there is a powdery deposit which disperses readily when shaken.

Biochemical tests

Indole,	+	Gelatin, growth but no liquefaction
Methyl red,	+	Nitrate, reduced to nitrite
Voges-Proskauer,	-	Urea hydrolysis, negative
Eijkman,	+	H_2S, negative
Citrate,	-	Litmus milk, acidity in 24 hr; clot within 48 hr

Fermentation tests

Acid and gas were produced from arabinose, fructose, galactose, mannitol, mannose, sorbitol, xylose, lactose, dulcitol and dextrose; the final pH in dextrose medium was 4.2. Adonitol, inositol and soluble starch were not attacked.

From the above characteristics the organism was identified as Escherichia coli using Berkey's Manual of Determinative Bacteriology (Breed et al., 1957). This strain of rumen E. coli was isolated in 1964 and will hereafter be referred to as E. coli 64.

Growth on different energy substrates

E. coli 64 grew well on glucose or lactate. With both substrates the lag phase was 4 hours. In the glucose medium exponential growth occurred during hours 5-8 of incubation; with lactate the exponential growth was slower but continued to approximately the 12th hour (Fig. 3). Cells grown aerobically in acetate or glycollate entered the logarithmic phase after a lag of 5-6 hr; exponential growth in the two carbon media was slower and the phase less clearly defined than in glucose or lactate media. At the end of 24 hr, however, the optical densities of acetate and glycollate cultures were only slightly lower than those of the glucose and lactate cultures.

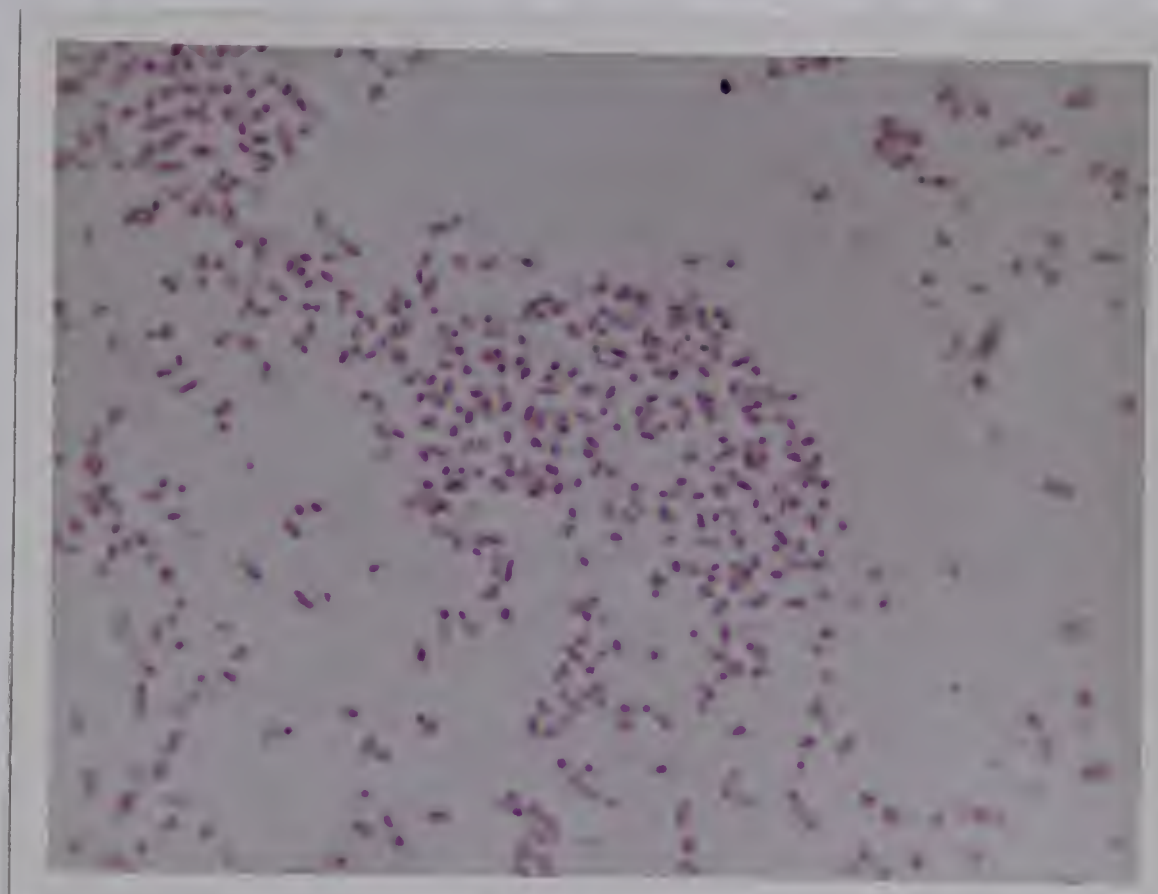


Fig. 2. Photomicrograph of rumen coliform isolate, Escherichia coli 64. ×1000.

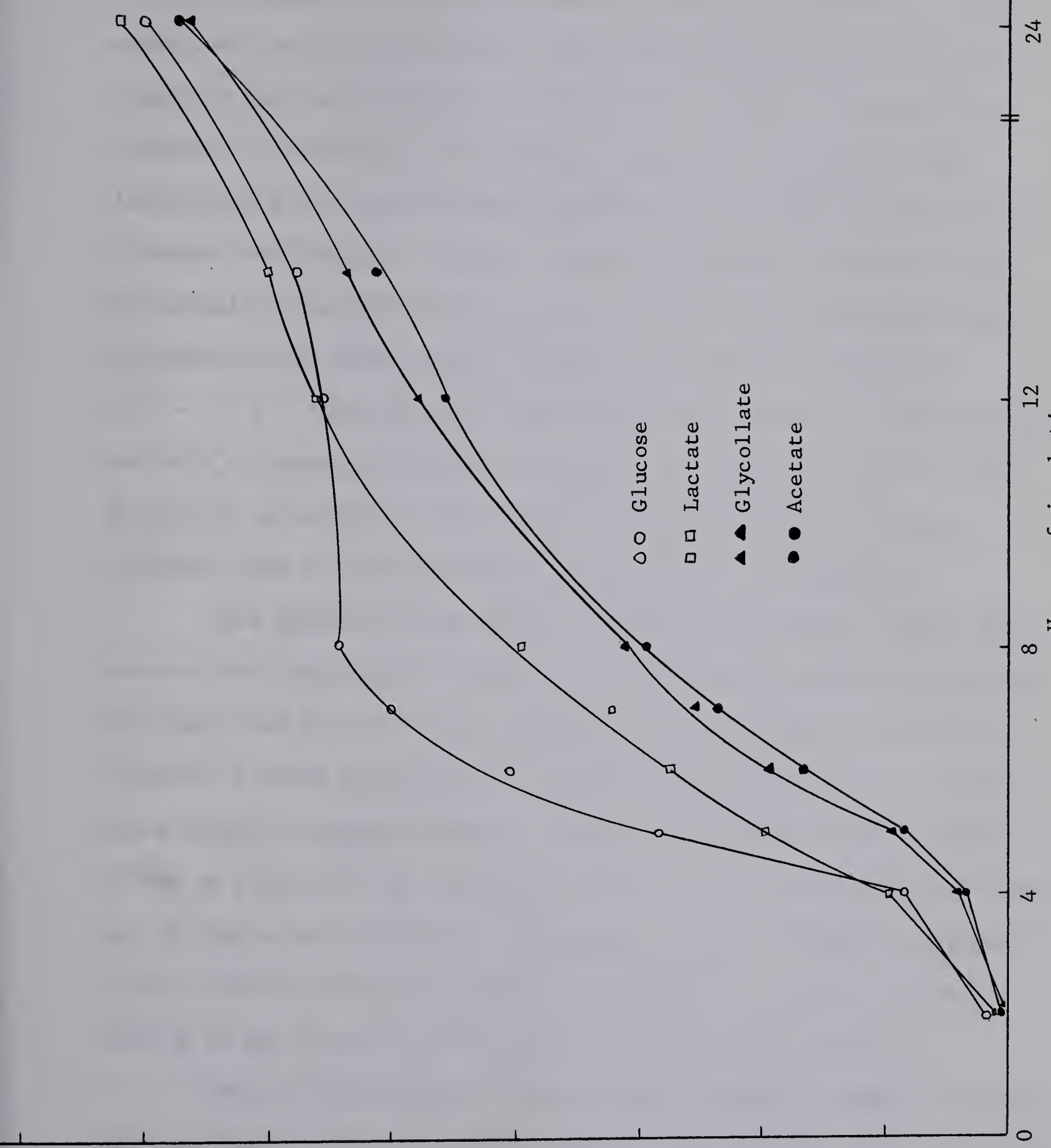


Fig. 3. Growth curves of E. coli 64 on different substrates.

Discussion

Identification of members of the family Enterobacteriaceae is difficult because of overlapping between several intermediate strains within well established groups. Hence Edwards and Ewing (1962) have suggested that more than one criterion must be used to type coliform organisms. Accordingly, the typing of E. coli 64 was based on the indole-methyl red-Voges-Proskauer-Eijkman-citrate (IMVEC) test as used by Mushin and Ashburner (1964). The only difference between this and the normally cited IMViC test is the addition of the Eijkman reaction. According to the IMVEC scheme, E. coli Type I gives the reactions (+ + - + -). Since E. coli 64 gave these reactions and in addition was not able to ferment adonitol or inositol, it is Type I. The fact that E. coli 64 is not able to use citrate as the sole source of carbon indicates that it is an intestinal rather than a soil organism.

This agrees with the result of Mushin and Ashburner (1964) who screened the Gram-negative aerobic flora in domestic animals in Australia and found that fecal and rectal swabs from calves, horses and pigs consistently yielded E. coli Type I, without even an association of other types of coli-aerogenes bacteria. Mann et al. (1954) studied in detail 18 out of a total of 150 isolates of rumen coliforms from sheep and found all of them to be intestinal type E. coli. Similarly Mackay and Oxford (1954) reported that 87% of their isolates from the rumen of calves varying in age from 10 to 105 days were E. coli Types I and II.

There is some question about whether coliforms commonly found in the rumen may be considered as true rumen microorganisms. This matter is of course important in relation to the hypothesis inherent in the present study that facultative anaerobes in general, and E. coli 64 in particular, may have a useful function in the rumen. As defined by Annison and Lewis

(1959) true rumen organisms are strictly anaerobic, present in a concentration of at least 10^6 per ml and are able to bring about chemical reactions characteristic of the rumen.

Hungate (1960) states that "facultative anaerobes are handicapped by carrying the genes necessary for synthesis of both anaerobic and unused aerobic enzymes and hence may not grow as fast in the rumen as their less encumbered anaerobic competitors." However, using rumen fluid from sheep fed hay, Heald et al. (1953) isolated numerous pure cultures of coliform bacteria capable of readily fermenting glucose and xylose and concluded that there are good reasons for believing that the rumen normally contains a considerable population of facultative anaerobes active in the fermentation of carbohydrates. Indirect evidence for the hypothesis that organisms other than obligate anaerobes may contribute to metabolism in the rumen was presented by Baldwin and Emery (1960), who bubbled oxygen through the rumen at a slow rate and found that it did not affect the redox-potential. They suggested that oxidized compounds that enter the rumen may be used by facultative organisms as hydrogen acceptors and that this may be responsible for maintaining the potential at a low level, thereby providing a better environment for the growth of anaerobes which constitute the major part of the rumen population. Heald et al. (1953) state that "preoccupation with cellulose fermentation which undoubtedly is an anaerobic process may have blinded some investigators to the fact that many other fodder carbohydrates, including soluble ones like sucrose and grass levans, are necessarily often fermented in large amounts in the rumen and that there is no reason at all to suppose that these other carbohydrate fermentations are brought about in the main by obligate anaerobic bacteria."

In Experiment 1 the mean number of viable coliforms in rumen fluid was of the order of 10^8 per milliliter. While this number is, of course, lower than that of the functionally more important cellulose digesting bacteria, it does satisfy one criterion for true rumen organisms, viz., that they be present in a concentration of at least 10^6.

The importance of certain non-cellulolytic bacteria may not depend as much on their relative numbers as on their special functions. For example, Peptostreptococcus elsdenii, which normally occurs in very small numbers in the rumen (Hobson et al., 1958), was found to occur in large numbers in steers suffering from feedlot bloat (Gutierrez et al., 1959). Because of its capacity to augment bloat symptoms, P. elsdenii is now regarded as an authentic rumen organism (Hungate et al., 1964). Inasmuch as the fate of lactate in the rumen is not fully understood, the capacity of E. coli 64 to utilize lactate to the same or even greater extent than glucose (Fig. 3) is interesting.

Acetate is the main end product of cellulose digestion in the rumen; thus growth of E. coli 64 on acetate suggests the possibility that coliform organisms may be concerned in metabolism of this important constituent of rumen fluid. Studies on acetate metabolism in E. coli 64 will be presented in Experiment 3.

Summary

1. A coliform organism was isolated from a dilution of 10^{-8} of rumen fluid obtained from a cow fed hay.
2. Morphological, cultural, biochemical and fermentative characteristics of this isolate were studied. Based on five tests, viz., indole, methyl red, Voges-Proskauer, Eijkman and citrate (IMVEC) the organism was typed as Escherichia coli Type I (rumen strain) and designated E. coli 64.

3. Growth characteristics of the organism on different substrates indicate that the rate of growth is faster with glucose and lactate than with acetate and glycollate.
4. Criteria proposed to characterize authentic rumen bacteria have been discussed in relation to the occurrence and properties of this coliform isolate.

Experiment 3
Utilization of acetate and carbon dioxide by *E. coli* 64

(i) Acetate

Introduction

Because volatile fatty acids are the main products of digestion of carbohydrates in the rumen, and acetic is under normal conditions the main VFA, the production and absorption of acetate have received extensive study and it is known that a large part of the acetate produced is absorbed directly from the rumen. Recent studies have indicated that acetate carbon may also be used for protein synthesis by rumen bacteria (Allison and Bryant, 1963; Hoover et al., 1963), methanogenesis (Oppermann et al., 1961) and for synthesis of long chain fatty acids (Keeney et al., 1962; Katz and Keeney, 1964). The fact that the molar proportions of VFA in the rumen remain relatively constant under given conditions of feeding suggests that a number of regulatory mechanisms are likely to be involved in maintaining this constancy. The increase in the number of coliform organisms immediately after feeding and the capacity of *E. coli* 64 to grow on 2C compounds, at least aerobically (Experiments 1 and 2), suggested that coliforms may be involved in the utilization of both fermentable carbohydrates and acetate in the rumen. With this in mind, the utilization of acetate by *E. coli* 64 was studied using labeled acetate.

Experimental

The details of the experimental procedures are given in Appendix p. 3-11.

E. coli 64 was cultivated by the method of Glasky and Rafelson (1959). After four serial transfers in an acetate medium, the cells were harvested in their logarithmic phase of growth and incubated for 1 hr with 1 μc of sodium acetate-2-C^{14}. At the end of incubation the cells were

separated from the medium by centrifugation, treated with hot ethanol and water and fractionated according to the method of Roberts et al. (1955), modified by deletion of the cold trichloracetic acid extraction step. This fraction contains most of the transient intermediates of the cell, all of which are extractable with ethanol. Inclusion of this step has the disadvantage that the trichloracetic acid must be removed to permit the use of chromatographic procedures.

The ethanol soluble supernatant was dried in vacuum and the residue treated successively with ethyl ether and distilled water to obtain the lipid and water soluble fractions of the cells. The aqueous portion was further fractionated into amino acids, organic acids and sugars by passing it through ion exchange resins (Canvin and Beevers, 1961). Radioactivity in organic acids and amino acids was detected by paper chromatography followed by radioautography (Aronoff, 1956). Radioactive counts on different fractions and identified compounds were done either in an end window gas flow counter* or in a liquid scintillation counting system**. $C^{14}O_2$ was counted either directly in the liquid scintillation system or, after conversion to barium carbonate, in the gas flow counter.

To study the sequence in which different cell constituents became labeled, a time course experiment was done. The experimental procedure was as outlined above except that samples were incubated with 0.50 μc of sodium acetate-2-C^{14}. Counting was done in the liquid scintillation system. Only the activities in the aqueous portions of the ethanol soluble, protein hydrolysate and CO_2 fractions of samples were recorded.

The use of ion exchange resins coupled with paper chromatography and radioautography greatly facilitated the identification of several

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*Nuclear Chicago, Model D-47, Ser-2072.
**Nuclear Chicago, Model 725.

compounds. The fractionation of the ethanol soluble portion into amino acids, organic acids and sugars is preferable to directly spotting the protein-free supernatant of the incubation mixture on paper, followed by radioautography as was done by Kornberg (1958). The present procedure simplifies the choice of specific solvents or solvent pairs because amino acids, organic acids and sugars are spotted on separate chromatograms.

Results

The distribution of radioactivity, after 1 hr of incubation with sodium acetate-2-C^{14}, shows that carbon dioxide and all the major cellular constituents were labeled in varying degrees (Table 4). One-third of the label added to the medium was detected in CO_2, which indicates that much of the acetate carbon was used for energy purposes. Of the radioactivity incorporated into cellular constituents, approximately 72% was recovered in the protein hydrolysate, 8% in the nucleic acid, 6% in the lipid and 14% in the aqueous fractions. Of the radioactivity in the aqueous fraction, 69% was contributed by organic acids and 16% by amino acids (see Appendix p. 20). No radioactivity was detected in the neutral sugar fraction. In summary, these results indicate, that of the labeled acetate added, approximately 33% was used to supply energy and 43% was incorporated into the cells.

The results for incorporation of radioactivity in individual amino acids of the protein hydrolysate show that 45-50% of the activity was present in aspartic and glutamic acids, with 5-10% in each of glycine, serine, alanine and threonine and traces in arginine, leucine and isoleucine (Table 5).

The radioautograms of the protein hydrolysates of the three replicates were identical. Figure 4 is a radioautogram of the protein hydrolysate from replicate 1.

Table 4. Recovery and distribution of radioactivity after incubation of E. coli 64 for 1 hr with sodium acetate-2-C^{14}

	Replicate 1	Replicate 2	Replicate 3	Avg
Counts per min in:				
CO_2	124,973	118,973	124,973	
Incubation mixture	214,500	201,480	236,120	
Total	339,473	320,453	361,093	
Total C^{14} added	370,000	370,000	370,000	
% of added C^{14} recovered in*:				
CO_2	34	32	34	33.3
Cells	41	43	44	42.7
Supernatant	15	10	19	14.7
Total	90	85	97	90.7
% of total activity in cells recovered in cell fractions:				
Protein	73	72	73	72
Nucleic acid	8	8	7	8
Lipid	6	7	6	6
Aqueous	13	14	15	14

*See Appendix p. 20.

Table 5. Distribution of radioactivity in amino acids (Replicate 1)

Amino acid	Activity count/min	% of total
Total	111,120	
Aspartic acid	27,174	25
Glutamic acid	23,672	21
Glycine	12,630	11
Serine	9,300	8
Alanine	5,658	5
Threonine	6,554	6
Arginine	760	1
Leucine + Isoleucine	736	1
Unidentified	24,636	22

Fig. 4. Radioautogram showing the distribution of radioactivity in protein hydrolysate after incubation of E. coli 64 with acetate-2-C^{14} for one hour.

The results of the time course study show that the percentages of radioactivity of the total label added that were recovered in carbon dioxide and protein hydrolysate increased with time (Table 6). The corresponding percentages for activity recovered in the supernatant decreased with time while those in organic acids and amino acids showed no definite changes. To illustrate the relative rates at which radioactivity was incorporated into different cell fractions the counts for the organic acid fractions and those for the protein hydrolysates were calculated as percentages of the total radioactivity in organic acids plus amino acids plus protein hydrolysate; the results are summarized in Fig. 5.

As early as 20 sec the radioactivity in the organic acids was approximately 63% of the total incorporated while that in the protein hydrolysate was only 3%. Most of the activity in the organic acid fraction was confined to the malic acid region when the chromatogram was run in the solvent n-propanol-formic acid-water, 8:1:1 by volume. The observed R_f value of 0.60 corresponded to standard malic acid in this solvent. There was a gradual fall in the activity of the organic acids with time while there was a steady increase in that of the protein hydrolysate which ultimately exceeded the former (Table 6, Fig. 5).

Table 6. Recovery and distribution of radioactivity at six intervals during incubation for 1 hr with sodium acetate-2-C^{14}

Fraction	Duration of incubation					
	20 sec	40 sec	60 sec	15 min	30 min	60 min
Counts per minute in:						
CO_2	26,400	74,400	108,260	178,320	247,740	354,840
Supernatant	709,820	668,504	555,460	361,176	212,940	65,640
Protein hydrolysate	1,720	3,560	32,160	114,780	196,740	250,320
Organic acids	33,660	28,560	54,660	66,240	37,020	32,400
Amino acids	18,400	11,520	11,580	56,640	48,240	26,400
Total	790,000	786,544	762,120	777,156	742,680	729,600
Total of C^{14} added	800,000	800,000	800,000	800,000	800,000	800,000
% of added label in:						
CO_2	3	9	14	22	31	44
Supernatant	89	84	69	45	27	8
Protein hydrolysate	0.2	0.4	4	14	25	31
Organic acids	4	4	7	8	5	4
Amino acids	2	1	1	7	6	3
Total	98.2	98.4	95	96	94	90
% of total activity in cells recovered in cell fractions:						
Protein hydrolysate	3	8	33	48	70	81
Organic acids	63	65	55	28	13	10
Amino acids	34	26	12	24	17	9

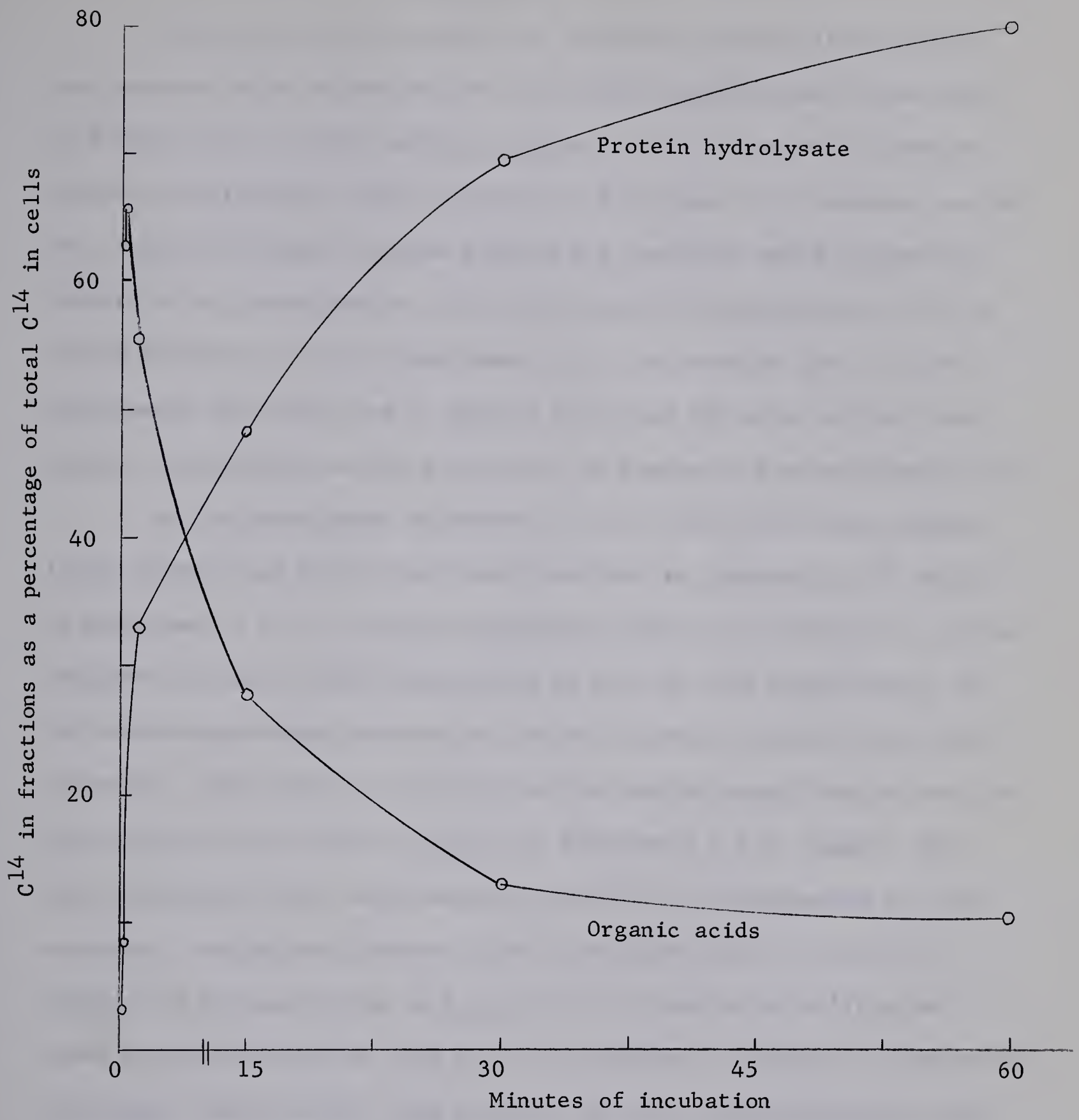

Fig. 5. Changes in per cent of total cellular radioactivity in organic acids and protein hydrolysates at intervals during one hour of incubation.

Discussion

The results for incorporation of labeled acetate into cellular constituents after incubation for 1 hr differ quantitatively from those of Roberts et al. (1955) with *E. coli* strain B. The major differences appear to be related to the fact that in Experiment 3 (i) acetate was the sole source of carbon, whereas Roberts and coworkers added radioactive acetate to a glucose medium. They observed 15% incorporation of C^{14} in carbon dioxide vs. 33% in Experiment 3 (i), but mention that in other experiments (not described in detail) $C^{14}O_2$ was the major product when acetate was supplied as the sole source of energy to glucose-adapted cells.

In the experiments of Roberts et al., 40% of the label appeared in the protein and 43% in the lipid fractions as compared to 72% and 6 % in Experiment 3 (i). In their experiments, cells could derive the carbon required for amino acids from glucose as well as from acetate while in the present experiment acetate was the only source of carbon for protein synthesis. The absence of activity in the neutral sugar fraction and the low activity in the lipid fraction, in Experiment 3 (i), suggest that the conditions of this experiment did not favor polysaccharide or lipid synthesis. Wright and Lockhart (1965) have shown that the cellular contents of polysaccharide in *E. coli* K-12 is greater in cells grown under nitrogen limitation with glucose available in excess. In the present experiment, carbon rather than nitrogen was the limiting nutrient in the medium. Presumably, in a carbon limiting medium, a larger proportion of the energy has to be directed towards cellular upkeep with resulting limited polysaccharide or lipid formation.

The fact that aspartic and glutamic acids were more heavily labeled than other amino acids suggests the probability that they are synthesized rather directly from acids formed from acetate in the Krebs

cycle. The curves in Fig. 5 show that the activity in the organic acid fraction is initially much higher than in the protein hydrolysate, is equal to it at about 8.5 min and becomes less thereafter. This is in accordance with the principles of precursor-product relationship as defined by Zilversmit et al. (1943).

Nevertheless, without conducting degradation studies of the amino acids formed and obtaining a knowledge of the labeling patterns as was done by Cutinelli et al. (1951), no definite conclusions may be drawn on the pathways involved in the utilization of acetate other than that acetate is utilized for both synthetic and energy purposes.

If the Krebs cycle were the only pathway involved, the rapid draining of the cycle intermediates to provide carbon skeletons of amino acids would, as suggested by Kornberg (1958), cause the cycle to stop, unless there were a net synthesis of C_4 acceptors from acetate. The next experiment was carried out to explore possible means by which replenishment of the drained intermediates may be brought about.

Summary

1. Incubation of whole cells of *E. coli* 64 with sodium acetate-2-C^{14} showed that about 33% of the label appeared in CO_2.
2. The distribution of radioactivity in the protein hydrolysates, nucleic acids, lipids and ethanol soluble fractions were 72, 8, 6 and 14% respectively. The results suggest that acetate was utilized both for synthetic and energy purposes.
3. The facts that aspartic and glutamic acids were more heavily labeled than other amino acids and the organic acids were the first to be labeled, followed by the protein hydrolysate, suggest that Krebs cycle or one of its modified forms may be operating in the utilization of acetate by this organism.

(ii) Carbon dioxide

Introduction

Following the recognition by Wood and Werkman in 1936 of the importance of CO_2 fixation in propionic acid bacteria, much work has been done on the mechanism involved in the process. Wood and Stjernholm (1962) have described 12 primary CO_2 fixing reactions in heterotrophic organisms. Ashworth et al. (1965) consider that, in microorganisms, the following reactions are at least theoretically capable of effecting the net formation of C_4 acids by carboxylation of C_3 acids:

1. Pyruvate + ATP + CO_2 ⟶ Oxaloacetate + ADP + Pi (Seubert and Remberger, 1961)

2. Pyruvate + $NADPH_2$ + CO_2 ⟶ Malate + $NADP^+$ (Ochoa et al., 1948)

3. Phosphopyruvate + CO_2 + H_2O ⟶ Oxaloacetate + Pi (Bandurski and Greiner, 1953)

4. Phosphopyruvate + CO_2 + ADP ⟶ Oxaloacetate + ATP (Utter and Kurahashi, 1954)

However, relatively little is known regarding the quantitative significance of these reactions to the overall carbon economy of cells. Roberts et al. (1955) found that, in E. coli, practically all the $C^{14}O_2$ incorporated could be accounted for in amino acids, purines and pyrimidines. Using $C^{14}O_2$, Abelson et al. (1952) found that about 50% of the tracer added to a glucose medium was fixed by E. coli strain B and that 0.21 mole of labeled CO_2 was fixed for each mole of glucose metabolized. Kornberg (1956) reported that as much as 70% of the carbon of amino acids synthesized by Pseudomonas KBI growing on acetate as the sole source of carbon may be derived from CO_2. In view of the importance of CO_2 fixation in heterotrophic organisms, Experiment 3 (ii) was done in an attempt to determine whether the production of dicarboxylic acids from CO_2 by E. coli 64, grown in a medium in which acetate was the sole source of carbon, was adequate to maintain the Krebs cycle.

Experimental

The first part of this experiment was based on the 'isotopic competition' procedure of Roberts et al. (1955). Procedures for growth, cultivation, harvesting and incubation of the cells were the same as for Experiment 3 (i) (see Appendix p. 3). The following treatments were employed:

Treatment	Acetate			Acetate+Bicarbonate		
Flask no.	1	2	3	4	5	6
Incubation time, min	15	30	60	15	30	60
Cell suspension in phosphate buffer (OD = 2.0)	1.40 ml			1.40 ml		
Phosphate buffer (0.1 M, pH 7.0)	0.45 ml			0.25 ml		
Unlabeled potassium acetate (10 μmoles)	0.10 ml			0.10 ml		
Sodium bicarbonate (20 μmoles)	--			0.20 ml		
Added at zero time						
Sodium acetate-2-C^{14} (0.5 μc, specific activity 1 μc/0.414 μmole)	0.05 ml			0.05 ml		

At the end of 15, 30 and 60 min of incubation, one flask from each treatment group was removed and the incubation was stopped by immersing the flasks in a dry ice-acetone bath. The activities in the protein hydrolysate, amino acid and organic acid fractions were determined by pipetting an aliquot into 15 ml of Polyether 611 and counting in the liquid scintillation system as in Experiment 3 (i).

In the second part of the experiment, 1.4 ml of buffer solution containing washed cells (OD = 2) which had been grown in an acetate medium were incubated for 60 min in Warburg flasks with either sodium acetate-2-C^{14} or $C^{14}O_2$ as the source of carbon. The uptake of labeled acetate was studied by the same procedure as in Experiment 3 (i). At zero time 0.05 ml of an aqueous solution containing 0.5 μc of sodium

acetate-2-C^{14} was added from the side arm. To study the uptake of $C^{14}O_2$, 0.05 ml of a solution containing 0.5 µc of sodium carbonate-C^{14}* (specific activity 1.6 µc/0.635 µmole) was placed in the side arm which was closed with a vacuum bottle cap. At zero time $C^{14}O_2$ was liberated by injecting 3 drops of 6 N HCl into the side arm through the rubber cap (for details see Appendix p. 4).

The radioactivity in the protein fraction after incubation for 60 min was determined using the gas flow counter.

Results

The results of the 'isotopic competition' experiment show that, after 15 min of incubation, the activity in the protein hydrolysate of Flask 1 (acetate) was 114,780 count/min whereas in Flask 4 (acetate + $NaHCO_3$) it was only 34,200 count/min or 30% of that of Flask 1. However, after 30 min, activity in the hydrolysate from the flask containing both sources of carbon had increased to 50% and, after 60 min, to 99% of that in the flask in which acetate was the sole source of carbon (Table 7 and Fig. 6). This shows that, particularly during the early stages of incubation, the rate of incorporation of C^{14} into protein was reduced in the presence of sodium bicarbonate in a concentration twice that of total acetate.

In Fig. 6, the C^{14} activity of the protein hydrolysate from the flask containing an excess of bicarbonate is shown as a percentage of the C^{14} activity in the hydrolysate from the flask in which acetate was the sole source of carbon. The results indicate that the rate of incorporation of C^{14} from labeled acetate was reduced in the presence of unlabeled bicarbonate, but inasmuch as the total radioactivities of the protein fractions were essentially the same for both treatments after 60 min of incubation, the excess bicarbonate did not appear to inhibit growth.

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*Donated by Dr. E. A. Cossins, Department of Botany, University of Alberta.

Comparison of the points at which the extrapolated (broken lines in Fig. 6) 'acetate' and 'acetate + bicarbonate' lines intersect the ordinate suggests that, of the protein synthesized infinitely near zero time, only about 1/10 as much contained C^{14} in the presence as in the absence of excess bicarbonate. These results suggest that bicarbonate carbon was incorporated into cell protein but do not provide any indication of the relative proportions in which acetate and bicarbonate carbon units may have been used. The results of the second part of the experiment show that, when washed cells were incubated in Warburg flasks with sodium acetate-2-C^{14} as the only source of carbon, the activity in the protein hydrolysate was approximately 20 times that of the protein hydrolysate of cells incubated with $C^{14}O_2$ as the carbon source (Table 8).

Table 7. Effect of unlabeled bicarbonate on the incorporation of labeled acetate by E. coli 64

Treatment	Acetate (A)			Acetate + Bicarbonate (A + B)		
Flask no.	1	2	3	4	5	6
Incubation time, min	15	30	60	15	30	60
	count/min					
Organic acids	66,240	37,020	32,400	67,080	56,520	37,380
Amino acids	56,640	48,240	26,400	51,120	37,800	22,800
Protein hydrolysate	114,780	196,740	250,320	34,200	97,980	247,980
Total	237,660	282,000	309,120	152,400	192,300	308,160
$\frac{\text{Total } C^{14} \text{ in protein hydrolysate (A + B)}}{\text{Total } C^{14} \text{ in protein hydrolysate (A)}}$	x 100			30	50	99

Table 8. Comparison of utilization of labeled acetate and $C^{14}O_2$ by E. coli 64

Substrate		Radioactivity in protein hydrolysate after 1 hr of incubation, count/min
Sodium acetate-2-C^{14}	1	61,720
	2	64,780
	Avg	63,250
$C^{14}O_2$	1	2,800
	2	3,220
	Avg	3,010

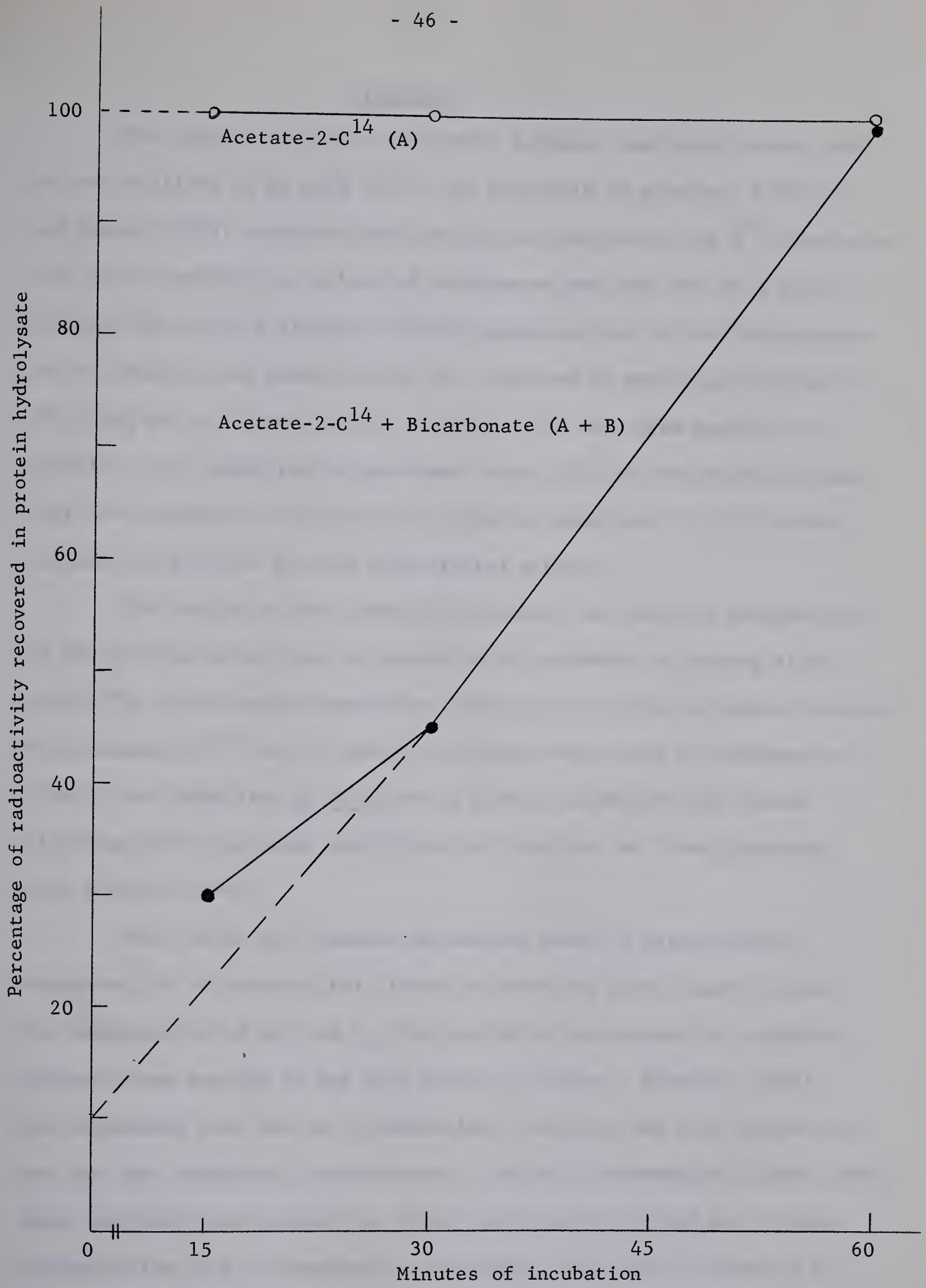


Fig. 6. Effect of unlabeled bicarbonate on the incorporation of labeled acetate by E. coli 64.

Discussion

The results of these experiments indicate that both acetate and CO_2 are utilized by E. coli 64 for the synthesis of protein. Kornberg and Quayle (1958) concluded that the initial suppression of C^{14} incorporation in the presence of unlabeled bicarbonate was due, not to a degree of fixation of CO_2 of a hitherto unknown magnitude, but to the "disturbance of the steady-state concentration of a compound in ready equilibrium with CO_2 lying on one of the earliest stages of the path from acetate to protein." By conducting an experiment over a 2.5 hr period, they showed that the presence of unlabeled CO_2 caused a reduction of 15-17% in the radioactivity of the protein from labeled acetate.

The results of the present experiment, in which the radioactivity of the protein hydrolysate of washed cells incubated in Warburg flasks with $C^{14}O_2$ as the carbon source was less than 5% of that of cells incubated with acetate-2-C^{14}, are in general agreement with those of Kornberg et al. (1960), who found that E. coli strain w cells incubated with sodium bicarbonate-C^{14} contained only 2% as much activity as those incubated with acetate-1-C^{14}.

Thus, while CO_2 fixation mechanisms exist in heterotrophic organisms, it is apparent that fixation reactions alone cannot provide for regeneration of all the C_4 dicarboxylic acids drained for synthetic purposes when acetate is the sole source of carbon. Krampitz (1961) has emphasized that the net condensation of acetate and a C_1 fragment has not yet been adequately demonstrated. Similarly Kornberg and Elsden (1961) have concluded that pyruvate is formed only indirectly and not through carboxylation of a C_2 compound. They suggest that the α carbon of C_2 compounds is transferred to a suitable acceptor which is also derived from C_2 substrate to form the β carbon of pyruvate.

The condensation of 2 molecules of acetate to form 1 molecule of succinate (Thunberg condensation) has long been regarded as a possible reaction by which C_4 acids are formed. Working with E. coli strain E26, Swim and Krampitz (1954b) provided evidence that the Thunberg condensation does not operate in the utilization of acetate. When the cells were incubated anaerobically with $C^{13}H_3COOH$ plus unlabeled fumarate to serve as an oxidant and to prevent recycling, they observed that only one of the methylene carbon atoms of the succinate formed contained C^{13}. This indicates that only the Krebs cycle operated under these conditions and, by inference suggests that it is improbable that the Thunberg condensation occurs under aerobic conditions.

On the other hand, from the results of kinetic studies, Glasky and Rafelson (1959) reported evidence in favor of the formation of succinate by Thunberg condensation. They found that the specific activity of succinate was initially higher than that of all other compounds when E. coli Crooks strain was incubated aerobically with acetate-1-C^{14} or 2-C^{14}. They therefore concluded that succinate was formed directly from acetate.

However, using the same and another strain of E. coli and acetate-1-C^{14}, Kornberg et al. (1960) found that citrate and malate were initially labeled and succinate was labeled only later and to a smaller extent. This is in general agreement with the results obtained with E. coli 64 in Experiment 3 (i).

The failure of Glasky and Rafelson (1959) to detect the presence of isocitritase in cells grown under conditions similar to those of Kornberg et al. (1960), coupled with the demonstration by Seaman and Naschke (1955) of the presence, in extracts of E. coli strain B and Streptococcus faecalis, of a succinate cleaving enzyme which reversibly

catalyzed the reaction acetyl-CoA $\rightleftharpoons$ succinate, suggests the possibility that other mechanisms may be active. The observation of French et al. (1964) that, after incubation of Micrococcus denitrificans with acetate-1-C^{14}, the label was found not only in C_1 and C_5 of glutamic acid, as would be the case if the Krebs cycle were the only pathway, but also in C_2, led them to conclude that unknown pathways may be involved in the oxidation of acetate by this organism.

A third pathway through which there can be a net synthesis of C_4 dicarboxylic acid is the glyoxylate cycle (Kornberg and Krebs, 1957). The following experiments were designed to study the possible presence and activity of the enzymes of the glyoxylate cycle in cell-free extracts of E. coli 64.

Summary

1. Results of 'isotopic competition' experiments indicate that the rate of incorporation of C^{14} from labeled acetate by E. coli 64 was reduced in the presence of unlabeled sodium bicarbonate, but the total radioactivity of the protein fractions, after 60 min of incubation, was essentially the same in the presence or in the absence of unlabeled bicarbonate. The results suggest that bicarbonate carbon was incorporated into cell protein but do not provide any indication of the relative proportions in which acetate and bicarbonate carbon units may have been used.
2. When washed cells of E. coli 64 were incubated with sodium acetate-2-C^{14} as the only source of carbon, the activity in the protein hydrolysate was approximately 20 times that of the protein hydrolysate of cells incubated with $C^{14}O_2$ as the carbon source.
3. It appears that CO_2 fixation reactions alone cannot provide for regeneration of all the C_4 dicarboxylic acids drained for synthetic purposes when acetate is the sole source of carbon.

ENZYMATIC STUDIES

Experiment 4
Assay of the enzymes of the acetate-activating system in cell-free extracts of *E. coli* 64

Introduction

Though it has long been recognized that acetate does not participate as such in biochemical condensations but only as "active acetate", the identification of the active compound as acetyl-CoA came only in 1951 through the work of Lynen and Reichert. Two acetate-activating systems have since been recognized. One is the acetyl-CoA synthetase system (systematic name, acetate:CoA ligase (AMP), EC 6.2.1.1), formerly known as aceto-CoA-kinase or acetyl thiokinase (Jones and Lipmann, 1955). This involves a pyrophosphoryl split of ATP which is coupled with the acetylation of CoA by free acetate,

ATP + acetate + CoA ⟷ AMP + pyrophosphate + acetyl-CoA.

The other system involves the reaction of ATP and acetate in the presence of acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) to form acetyl phosphate (Rose et al., 1954). The latter is then converted to acetyl-CoA by the action of phosphate acetyltransferase (Acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8 (Stadtman, 1952)). The steps involved are as follows:

ATP + acetate ⟷ ADP + acetyl phosphate

Acetyl phosphate + CoA ⟷ Acetyl-CoA + orthophosphate

It was considered desirable to demonstrate the presence of an acetate-activating system in cell-free extracts of *E. coli* 64 before assaying for the enzymes of the glyoxylate cycle.

Experimental

Cell-free extracts (CFE) of *E. coli* 64 grown on the acetate medium were prepared by the procedure outlined by Seaman (1963) - see

Appendix p. 11 for details. The protein concentration in CFE was determined from the absorbancies at 280 mμ and 260 mμ as described by Layne (1957).

The assay of acetate kinase was performed by the method of Seaman (1963) in which one unit of acetate kinase activity is defined as that amount which yields 1 μmole of hydroxamic acid per min. Phosphate acetyltransferase was measured by the method described by Stadtman (1955); in the present experiment one unit of phosphate acetyltransferase activity is the amount required to catalyze the arsenolysis of 1 μmole of acetyl phosphate per min. Enzyme assays were done on extracts which had been rendered low in CoA by adsorption on anion exchange resin.

Results

The concentration of protein in the CFE was 5.67 mg/ml (Table 9).

Table 9. Protein content of cell-free extracts of E. coli 64

Replicate	Absorbancy 280 mμ	Absorbancy 260 mμ	mg protein per ml diluted CFE*	CFE dilution	mg protein per ml CFE
I	0.562	0.980	0.8401 − 0.7448 = 0.0953	1:60	5.718
II	0.540	0.978	0.8370 − 0.7433 = 0.0937	1:60	5.622
Avg					5.67

*mg protein/ml = $1.55 \times A_{280} - 0.76 \times A_{260}$ (Layne, 1957)

The results of the acetate kinase assay show that in all 4 replicates the amount of acetyl hydroxamate formed was the same in the absence or presence of added CoA (Table 10). If aceto-CoA kinase had been involved in the activation process, addition of CoA to the CoA-deficient extracts should have increased the amount of acetyl hydroxamate formed. The fact that added CoA did not have any effect on the amount

of acetyl hydroxamate formed indicates that only acetate kinase was present in the extracts. The specific activity of acetate kinase was 2.54 units/mg protein.

In the assay of phosphate acetyltransferase (Table 11), the quantity of acetyl phosphate left after a portion had been converted to acetyl CoA by the action of phosphate acetyltransferase present in the extracts is indicated by the absorbancy of the acetyl hydroxamate in the tubes containing CFE. The absorbancy in tubes to which CFE was not added corresponds to the total amount of acetyl phosphate added to the reaction mixture in the form of its hydroxamate. The difference between readings reflects the amount of acetyl phosphate converted to acetyl CoA. Phosphate acetyltransferase had a specific activity of 2.50 units/mg protein.

Discussion

The results are in agreement with many reports in the literature which indicate that acetate kinase is most common in bacteria whereas aceto-CoA-kinase is most common in higher plants, birds and animals (Rose, 1962). The specific activity of acetate kinase in E. coli 64 is comparable to the value of 3 units reported by Rose et al. (1954) in E. coli strain 4157.

The fact that the specific activities of acetate kinase and phosphate acetyltransferase were the same indicates that in E. coli 64, as in most other bacteria, acetate is activated by a two-step process involving acetate kinase and phosphate acetyltransferase.

Table 10. Specific activity of acetate kinase in cell-free extracts of E. coli 64

	Acetyl hydroxamate (A_{540} treated tube-blank after addition of $FeCl_3$ reagent)		Specific activity* (units/mg protein)
Treatment	—CoA	+ CoA	
Replicate			
1	0.408	0.406	2.55
2	0.406	0.402	2.53
3	0.406	0.404	2.54
4	0.404	0.404	2.54
Avg			2.54 ± 0.005

$$*\text{Specific activity} = \frac{\text{Absorbancy x Dilution}}{0.14 \times 20 \times \text{mg protein/ml}}$$

Table 11. Specific activity of phosphate acetyltransferase in cell-free extracts of E. coli 64

	Acetyl hydroxamate (A_{540} treated tube-blank after addition of $FeCl_3$ reagent)		Specific activity* (units/mg protein)
Treatment	+ CFE	— CFE	
Replicate			
1	0.025	0.450	2.49
2	0.026	0.450	2.49
3	0.024	0.446	2.50
4	0.020	0.446	2.52
Avg			2.50 ± 0.016

$$*\text{Specific activity} = 6 \times \frac{\text{Absorbancy of tube without CFE} - \text{Absorbancy of tube with CFE}}{0.2 \times \text{Absorbancy of tube without CFE} \times \text{mg protein/ml}} \times \text{dilution}$$

Summary

1. The presence of acetate kinase and phosphate acetyltransferase in CFE of E. coli 64 was demonstrated.
2. The specific activities of the two enzymes were 2.54 and 2.50 units/mg protein respectively.
3. The results indicate that acetate was activated by the combined action of these enzymes.

Experiment 5
Assay of the enzymes of the glyoxylate cycle

Introduction

The enzymes of the glyoxylate cycle may be assayed by two basic procedures. Dynamic assays involve following the changes in the absorbancy at particular wavelengths associated with the characteristic absorption maxima of new compounds formed during the reaction. Static assays are based on the distribution of radioactivity or the absolute quantity of new compounds formed. Dynamic and static assays for enzymes of the glyoxylate cycle in CFE of E. coli 64 were done in Experiments 5 and 6.

Experimental

Preparation of CFE

The procedure for the preparation of CFE was essentially the same as in Experiment 4. After 4, 8, 16 or 24 hr of incubation the cells grown aerobically on acetate were harvested by centrifugation at 0 C in batches of 2000 ml in a Servall centrifuge at 15,000 rpm for 30 min. For comparative purposes, assays were done on extracts of cells grown aerobically on glucose using the same medium, except that 0.5% dextrose (Difco) was substituted for acetate, and on extracts of cells grown anaerobically on glucose with cysteine hydrochloride (100 mg/100 ml) and resazurin (0.1 mg/100 ml) added to the medium.

The assay of isocitrate lyase was done by the method of Dixon and Kornberg (1959) as modified by Megraw and Beers (1964) - see Appendix p. 14 for details. After demonstrating the presence of isocitrate dehydrogenase by the addition of isocitrate and $NADP^+$ and observing the increase in absorbancy at 340 mμ with time, the reversibility of the isocitrate lyase reaction was demonstrated by a method similar to that used by Kornberg and Madsen (1958). This method is based on the increase in the

absorbancy at 340 mμ by $NADPH_2$ formed from added $NADP^+$ by the action of isocitrate dehydrogenase on isocitrate arising from glyoxylate and succinate.

The specific activities of isocitrate lyase after 4, 8, 16 and 24 hr of incubation were determined from the amount of glyoxylate formed using the method of Daron and Gunsalus (1962) with slight modifications. In the estimation of glyoxylic acid, 0.05% instead of 0.2% 2,4-dinitrophenylhydrazine (DNP) in 2 N HCl was used. The 0.2% concentration gave a deep brown color too intense for small differences to be observed. The concentration of 2,4-DNP used and the time intervals employed before reading the color developed in the spectrophotometer yielded reproducible results - see Appendix p. 15 for details. One unit of isocitrate lyase activity is defined as that amount of the enzyme which catalyzes the formation of 1 μmole of glyoxylate/mg protein in 10 min at 30 C.

Malate synthase was assayed by the method of Dixon and Kornberg (1959) with minor modifications - see Appendix p. 17.

Results

Isocitrate lyase

After the addition of isocitrate to the reaction mixture, there was, in the presence of an extract of cells grown on acetate, a linear increase with time in the absorbancy at 324 mμ (Fig. 7). This increase is attributable to the presence of glyoxylic phenylhydrazone and is a measure of the amount of glyoxylate formed from isocitrate in the presence of isocitrate lyase. In the absence of CFE no increase in absorbancy was observed. Also there was a greater response when increasing quantities of CFE were added to the reaction mixture.

The enzyme could not be demonstrated in CFE of cells grown on glucose aerobically or anaerobically.

The presence of isocitrate dehydrogenase in CFE of E. coli 64 is shown by the increase in the absorbancy (Fig. 8) associated with the formation of $NADPH_2$ from the added $NADP^+$ as follows:

$$D_s \text{ - isocitrate} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{Isocitrate}} \text{2-oxoglutarate} + CO_2 + NADPH_2$$

The reversibility of the isocitrate lyase reaction was demonstrated by the fact that $NADP^+$ was reduced when glyoxylate and succinate were added to the reaction mixture, indicating that isocitrate was formed by the condensation of glyoxylate and succinate. Reduction of $NADP^+$ was not observed when glyoxylate or succinate was added separately (Fig. 9).

The specific activities of isocitrate lyase expressed as μmoles of glyoxylate formed per mg protein in 10 min show that a plateau was reached after 8 hr (Table 12). From results obtained in Experiment 2 (Fig. 3, p. 26) it seems reasonable to conclude that at 8 hr the cells were in the late exponential phase of growth.

Malate synthase

After the addition of glyoxylate to the assay system for malate synthase in extracts of cells grown on acetate, there was a decrease with time in the absorbancy at 232 mμ (Fig. 10), indicating the cleavage of the thiol ester bond of acetyl-CoA and probable concomitant synthesis of malate from glyoxylate and acetyl-CoA. The presence of malate in the protein-free supernatant was confirmed by paper chromatography using n-propanol-formic acid-water, 8:1:1 by volume as the solvent.

The absence of malate synthase in cells grown on glucose is indicated by the fact that no decrease in absorbancy was observed when CFE of such cells were assayed.

Table 12. Specific activities of isocitrate lyase in cell-free extracts prepared from samples taken from a culture of E. coli 64 after 4, 8, 16 and 24 hr of incubation

Hours	Replicate	A_{540}	Glyoxylate μg	Protein content of CFE $(1.55 \times A_{280}) - (0.76 \times A_{260})$ mg/ml	Specific activity*
4	1	0.015	12.8	1.74	0.645
	2	0.018	19.2		0.665
8	1	0.084	76.8	5.59	1.205
	2	0.073	75.5		1.185
16	1	0.071	65.3	4.60	1.220
	2	0.072	65.3		1.220
24	1	0.114	103	7.10	1.273
	2	0.110	103		1.265

*Specific activity = μmoles glyoxylate/mg protein/10 min

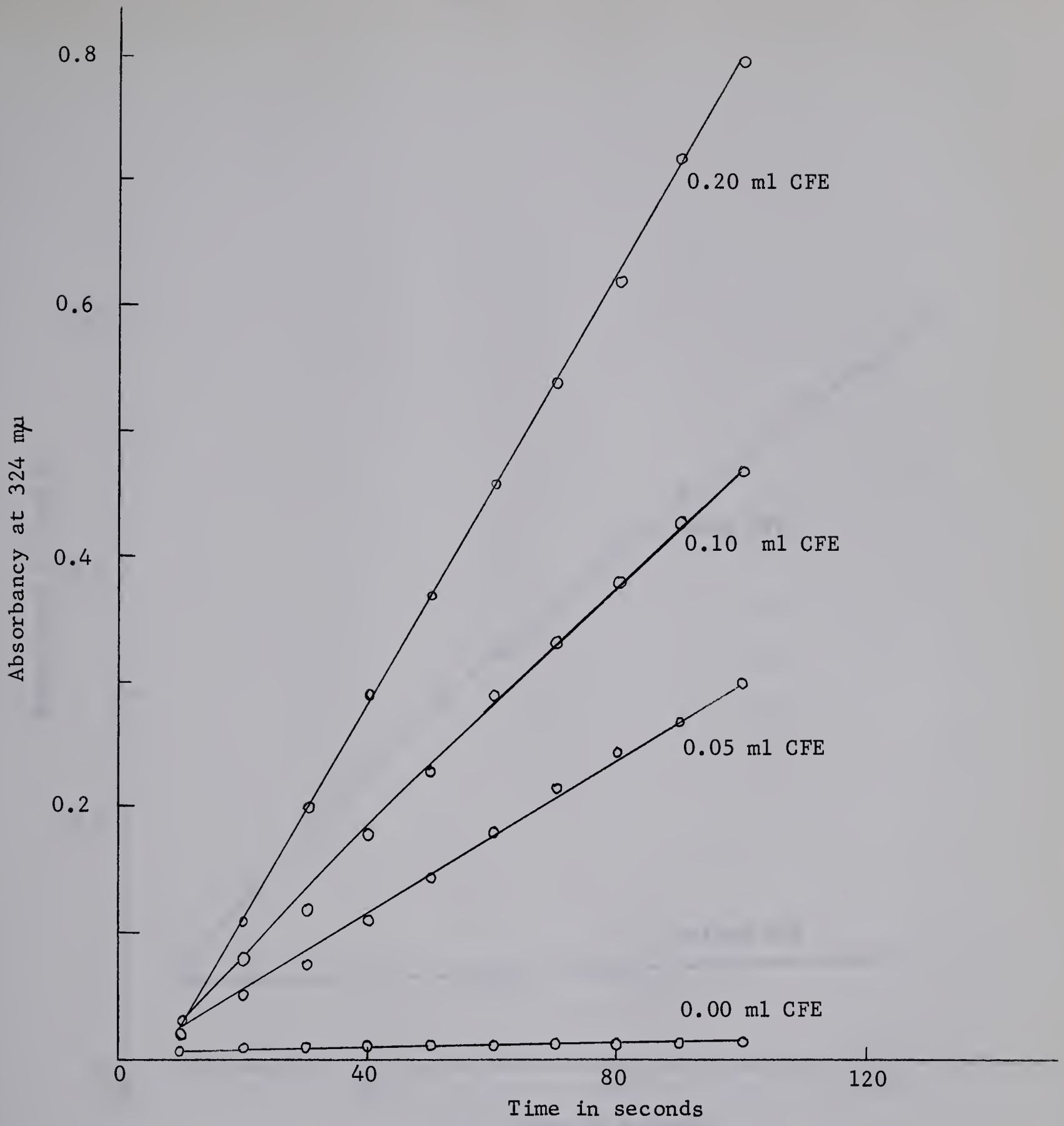
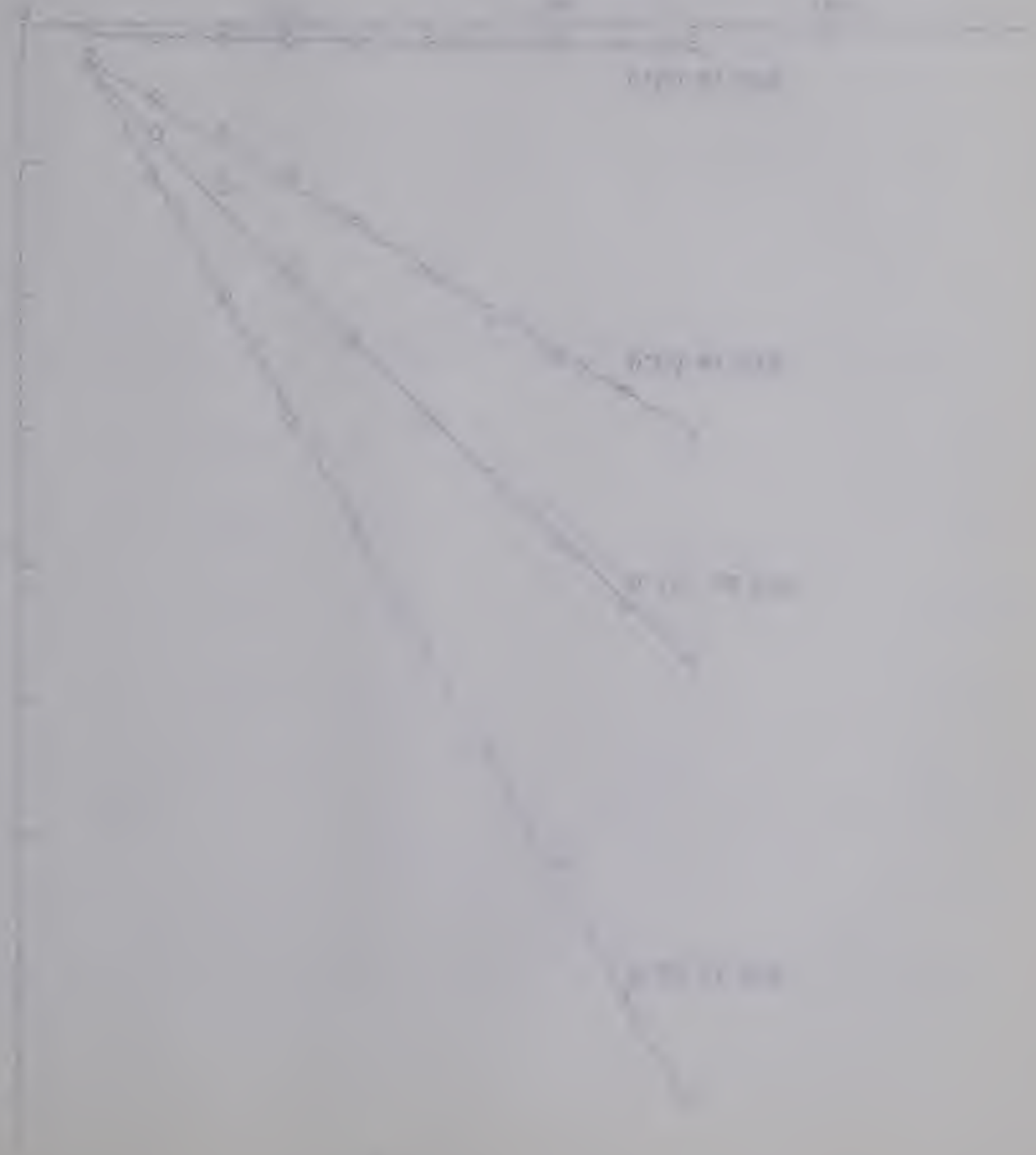


Fig. 7. Isocitrate lyase activity in CFE of E. coli 64 as shown by increase in absorbancy at 324 mμ associated with the formation of glyoxylate phenylhydrazone. The reaction mixture contained: 0.50 ml of 0.2 M Tris buffer, pH 7.2; 0.50 ml of 2.860% $MgCl_2.6H_2O$; 0.50 ml of 0.216% phenylhydrazine hydrochloride; 0.50 ml of 0.1454% cysteine hydrochloride; CFE, as indicated; distilled water to a total volume of 3.0 ml. At zero time, 5 μmoles of sodium isocitrate were added.



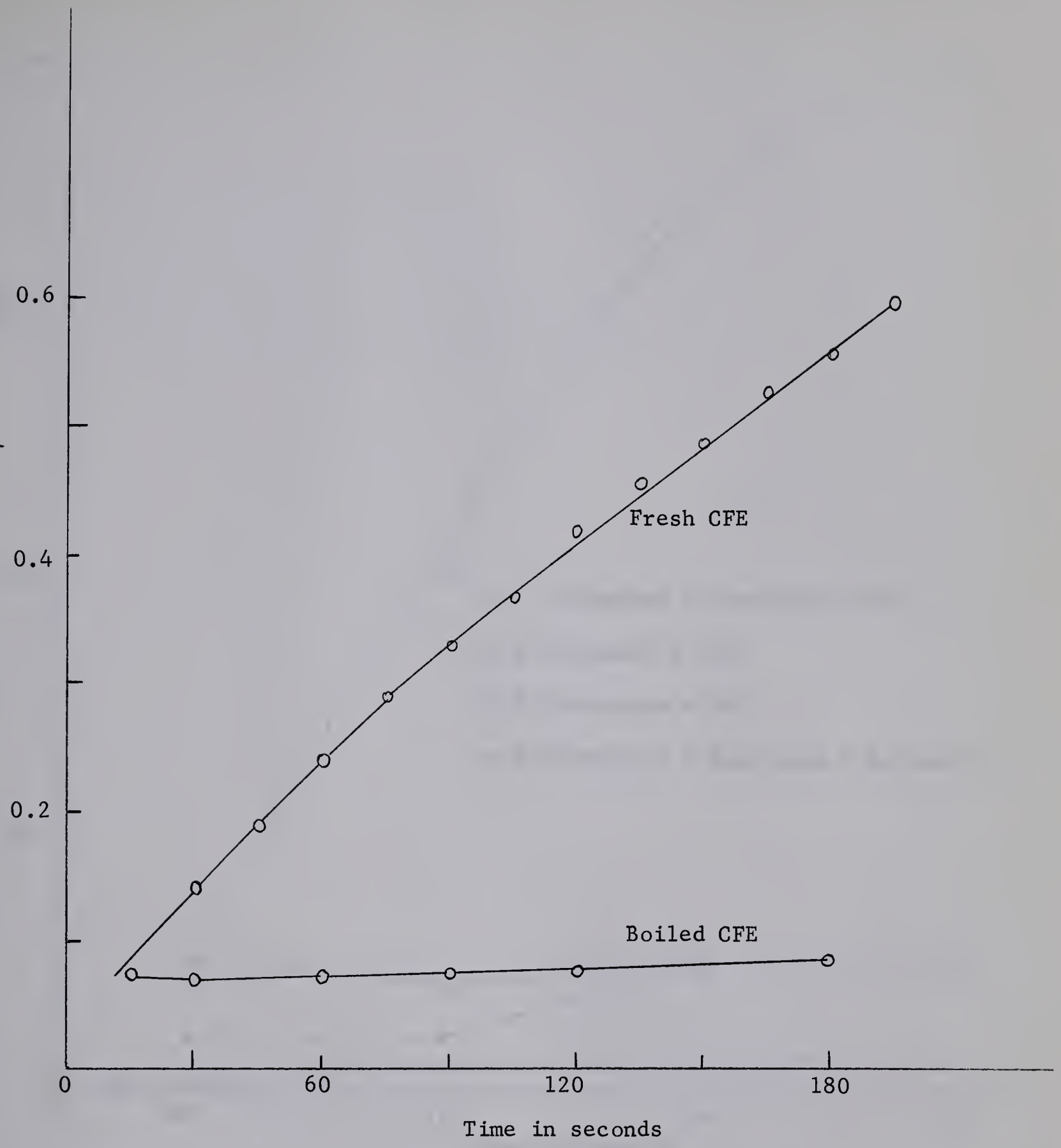


Fig. 8. Isocitrate dehydrogenase activity in CFE of E. coli 64 as shown by increase in absorbancy at 340 mμ associated with the formation of $NADPH_2$ from $NADP^+$. The reaction mixture contained: 0.50 ml of 0.2 M Tris buffer, pH 7.2; 0.50 ml of 2.860% $MgCl_2.6H_2O$; 0.50 ml of 0.1454% cysteine hydrochloride; 0.20 ml of 0.554 μM $NADP^+$; 0.05 ml of CFE; distilled water to a total volume of 3.0 ml. The reaction was started by the addition of 5 μmoles of sodium isocitrate at zero time.

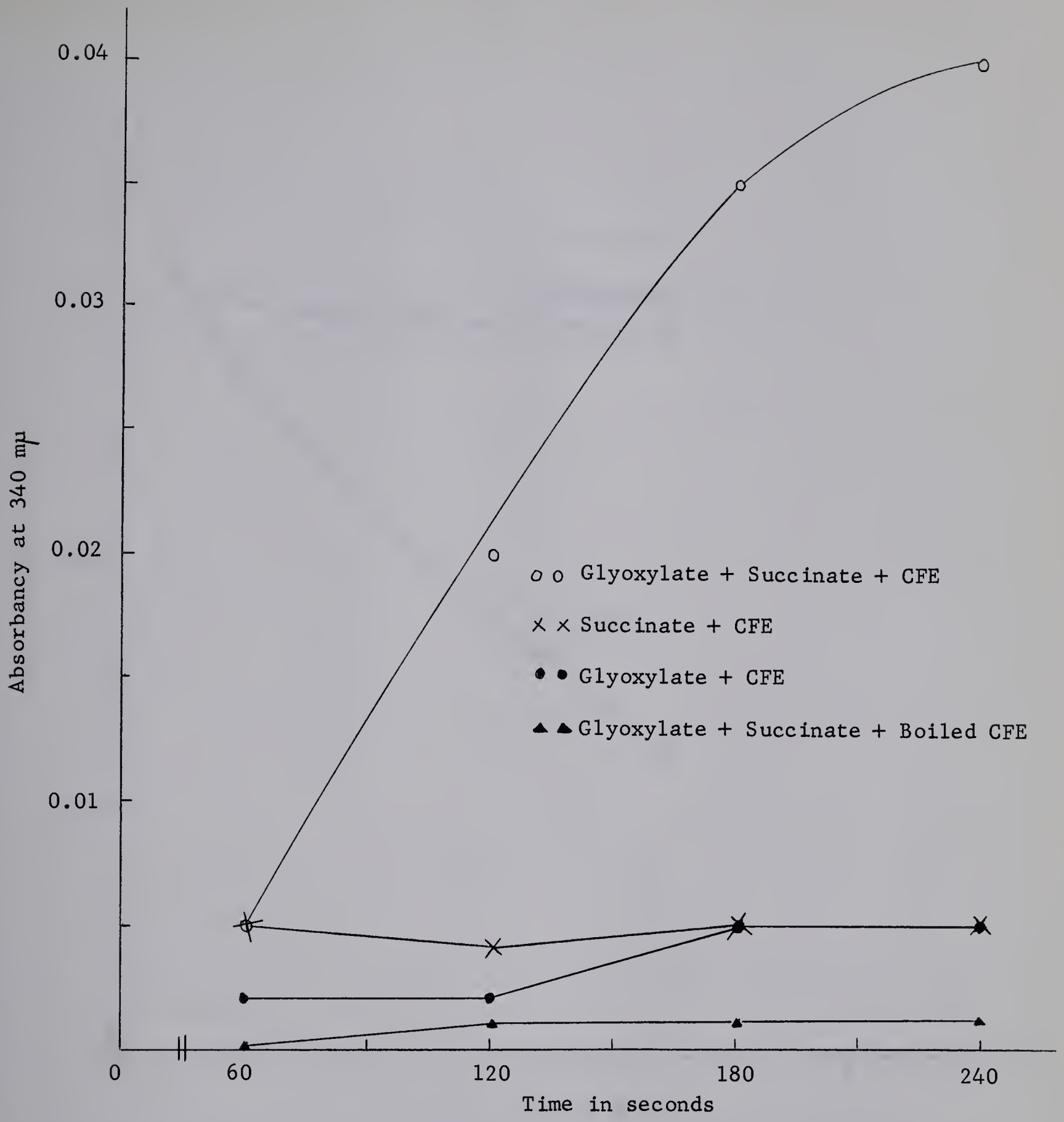
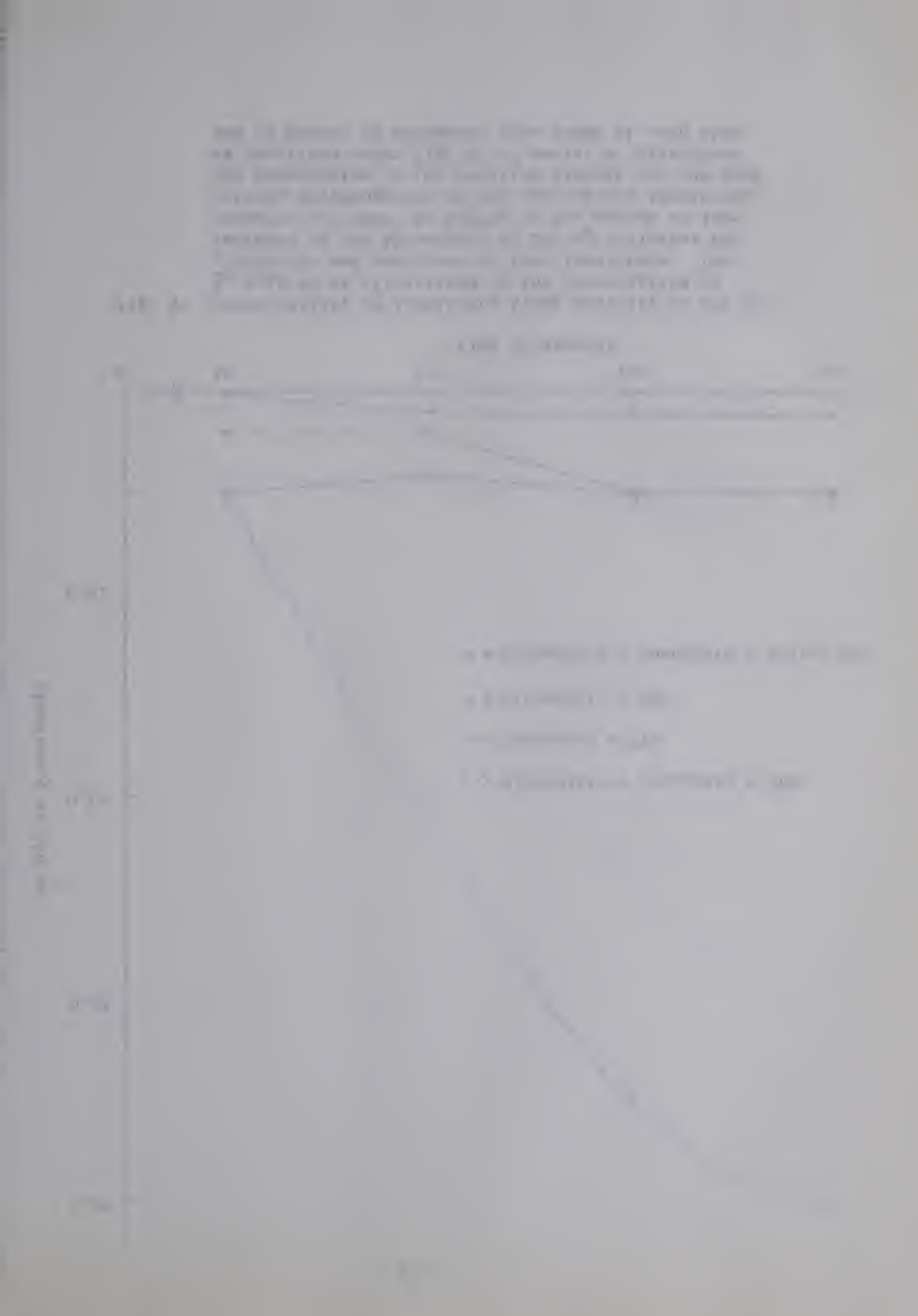


Fig. 9. Reversibility of isocitrate lyase activity in CFE of E. coli 64 as illustrated by the condensation of glyoxylate and succinate to form isocitrate. The increase in the absorbancy at 340 mμ indicates the reduction of $NADP^+$ to $NADPH_2$ by the action of isocitrate dehydrogenase on the synthesized isocitrate. The composition of the reaction mixture was the same as described under Fig. 8; 20 μmoles of glyoxylate and 10 μmoles of succinate were added at zero time.



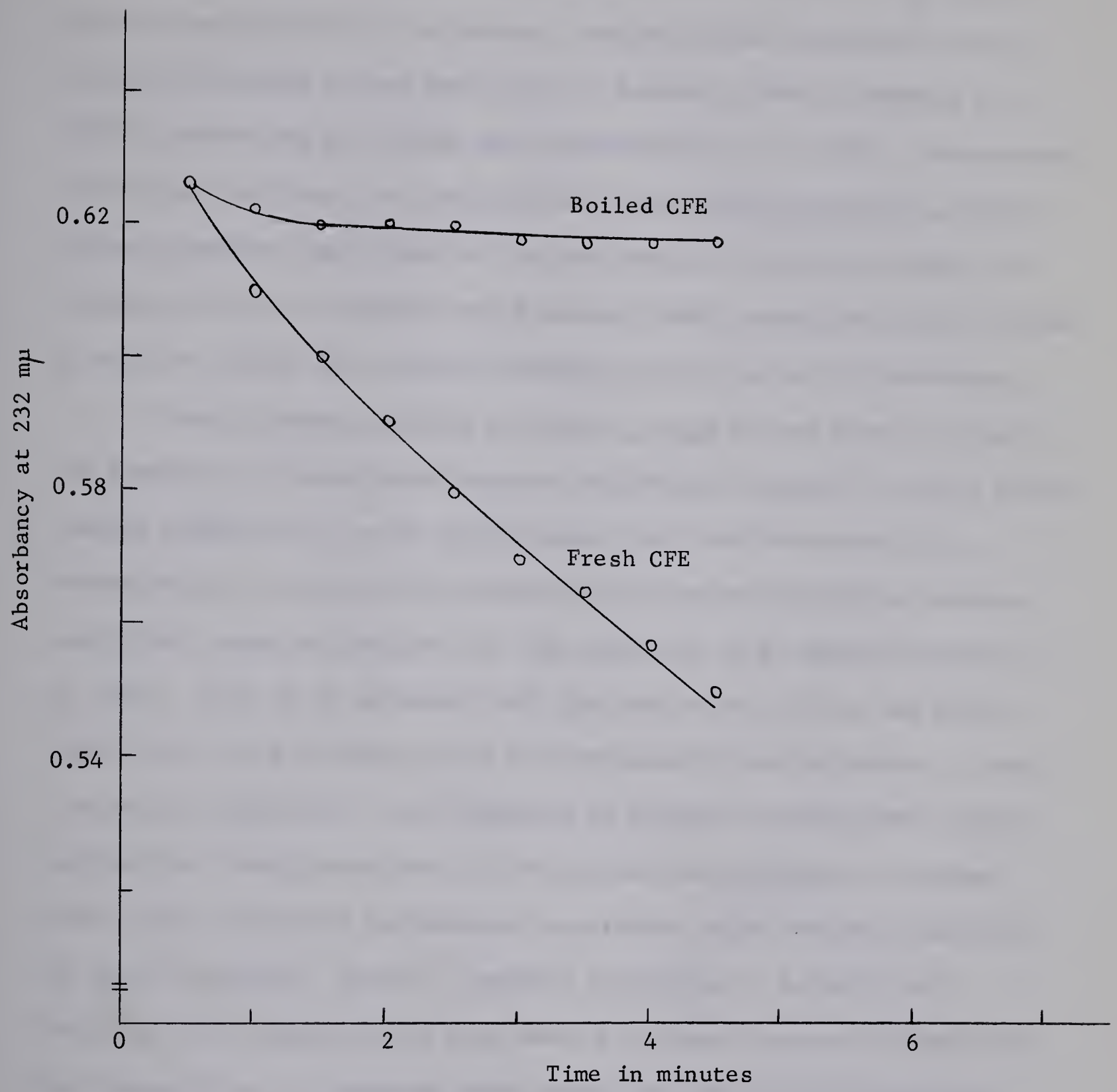
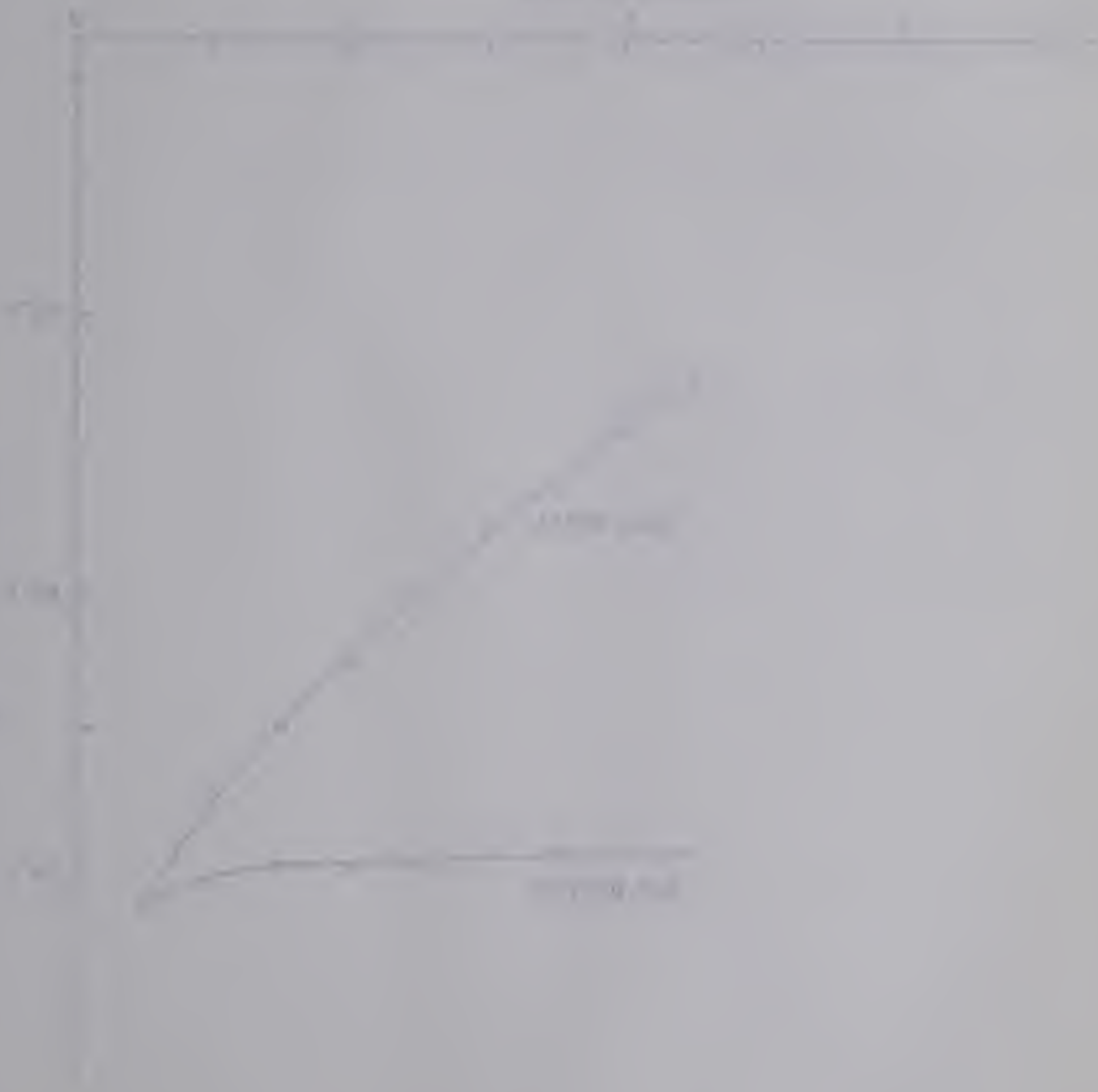


Fig. 10. Malate synthesis from glyoxylate and acetyl-CoA is indicated by a decrease in absorbancy at 232 mμ associated with breakage of the thiol-ester bond of acetyl-CoA. The reaction mixture contained: 0.50 ml of 0.1 M Tris buffer, pH 8.0; 0.07 ml of 15 μM $MgCl_2.6H_2O$; 0.05 ml of CFE; distilled water to a total volume of 3.0 ml. At zero time 0.02 ml of 2 μM sodium glyoxylate was added to the reaction mixture.



Discussion

The fact that isocitrate lyase was present in CFE of *E. coli* 64 grown on acetate but not on glucose, indicates that isocitrate lyase is an adaptive enzyme as has been shown by Kornberg (1960), Kornberg et al. (1958), Reeves and Ajl (1960) and Vanderwinkel et al. (1963). Rosenberger (1962) reported that, in a tartrate-utilizing *Achromobacter sp.*, acetate had an induction-like effect on the synthesis of isocitrate lyase, but Kornberg (1961) and Ashworth and Kornberg (1963) demonstrated that acetate is not the inducer and that the feedback control is one of repression.

Thus it seems probable that when *E. coli* 64 was grown on glucose, the synthesis of isocitrate lyase was suppressed, because the cells could readily synthesize C_4 acids from glucose, but that this repressing mechanism was not active when the cells were grown on acetate, because isocitrate lyase was required for the synthesis of an adequate supply of C_4 acids. This is in agreement with the conclusion of Maas and McFall (1964) that the glyoxylate cycle is controlled by two mechanisms. First, 'catabolite repression' - as suggested by Neidhart and Magasanik (1957), catabolites from glucose are able to repress the *synthesis* of enzymes which would only serve to increase the already large intracellular pools of these compounds. Second, 'feedback inhibition' - Ashworth and Kornberg (1963) demonstrated that added phosphoenol pyruvate impedes the net formation of C_4 compounds from acetate by inhibiting the *activity* of isocitrate lyase.

The increase in absorbancy observed in the assay done to demonstrate the synthesis of isocitrate from glyoxylate and succinate in the presence of isocitrase lyase (Fig. 9) was less than one-tenth that observed in the assay conducted to demonstrate the cleavage of isocitrate to glyoxylate and succinate (Fig. 7). These results are in agreement

with the conclusion of Kornberg and Elsden (1961) that, under physiological conditions, the enzyme catalyzes cleavage rather than synthesis of isocitrate, even when glyoxylate and succinate are not metabolically removed. The fact that an increase was observed in the specific activity of isocitrate lyase during the early periods of incubation is in agreement with the findings of Howes and McFadden (1962a) with Pseudomonas indigofera and those of Megraw and Beers (1964) with Bacillus cereus.

The absence of malate synthase in CFE of E. coli 64 grown on glucose is in accordance with the findings of Krampitz (1961) with Micrococcus lysodeikticus and of Wong and Ajl (1957) with E. coli strain E26. Kornberg and Elsden (1961) have suggested that, though malate synthase has been found in several organisms under different conditions of growth, the enzyme may, in certain organisms, rather than being constitutive, be induced by glyoxylate formed from isocitrate. The fact that malate synthase was found in cells of E. coli 64 grown on acetate, but not in those grown on glucose, suggests that formation of the enzyme was induced. However, the possibility that malate synthase may be constitutive in this organism is not eliminated because very low levels of the enzyme in cells grown on glucose may not have been detected by the spectrophotometric method employed.

The presence of malate synthase in E. coli strain E26 grown on glucose has been explained by Reeves and Ajl (1960) on the assumption that some glyoxylate was formed from glucose. It is difficult to visualize a function for malate synthase in bacteria grown on glucose, but malate synthase is obviously necessary for growth on acetate or compounds more oxidized than acetate.

A possible explanation for the range in level of specific activities of malate synthase and isocitrate lyase reported in the literature could be

that most of the data on specific activities of these enzymes have been derived from experiments in which either the disappearance of the substrates or the formation of the products of the glyoxylate cycle was considered. Recent observations have revealed that malate synthase is involved in other metabolic pathways. As one example, organisms growing on C_2 compounds more oxidized than acetate employ the glycerate pathway (Kornberg and Sadler, 1961) wherein malate synthase serves to accomplish the net formation of 1 mole of malate from 3 moles of glycollate (see page 11). Also, though the malate synthase reaction is irreversible (Dixon et al., 1960), malate in Rhodopseudomonas spheroides has been shown (Tuboi and Kikuchi, 1965) to combine with ATP and CoA to yield malyl-CoA, ADP and Pi as follows,

$$\text{malate} + \text{ATP} + \text{CoA} \xrightarrow{\text{1-malate:CoA ligase (ADP)}} \text{malyl-CoA} + \text{ADP} + \text{Pi},$$

acetyl-CoA and glyoxylate are then formed from malyl-CoA,

$$\text{malyl-CoA} \xrightarrow{\text{1-malyl-CoA lyase}} \text{acetyl-CoA} + \text{glyoxylate}.$$

Further, the condensation of glyoxylate with acetyl-CoA to yield malate is only one of several reactions in which glyoxylate may participate. As examples, in addition to condensing with acetyl-CoA, glyoxylate has been shown to condense with other short-chain acyl-CoA derivatives (Wegener et al., 1965); Bolcato et al. (1958) have proposed a pathway of acetate oxidation in yeast through glycollate, glyoxylate and eventually to CO_2 and H_2O; the presence of glycollic oxidase and glyoxylic reductase has been demonstrated in bacteria by Katagiri and Tochikura as cited by Hassall and Hullin (1962).

Hence, while the feedback regulation of isocitrate lyase depends ultimately on the supply of C_4 acids, the multifarious reactions involving malate and glyoxylate are jointly responsible for the regulation of malate

synthase. Though malate synthase may be constitutive, its level is dependent on many factors, including growth substrate, growth rate, which determines the relative rates at which carbon is used for energy or synthesis, and the influence of other enzymes that are capable of contributing to the metabolism of glyoxylate or malate. An investigation by Howes and McFadden (1962b) on the differential rates of synthesis of malate synthase and isocitrate lyase during different phases of growth indicated that separate control of each of these enzymes exists in Ps. indigofera. A recent study by Falmagne et al. (1965) has demonstrated the existence of two malate synthases involved in the use of acetate and glycollate respectively. Conclusions regarding specific control mechanisms for malate synthase must be interpreted with caution when crude cell-free extracts are employed.

Summary

1. The presence of isocitrate lyase and malate synthase in CFE of E. coli 64 cells grown on acetate was demonstrated by spectrophotometric methods.
2. Neither enzyme was present in extracts of cells grown on glucose.
3. Factors governing the synthesis and activity of these enzymes have been discussed in relation to variations in their levels in crude cell-free extracts under different conditions.

Experiment 6
Isotope studies on enzymes of the glyoxylate cycle

Introduction

Demonstration, by dynamic assays, of the presence of the enzymes of the glyoxylate cycle in extracts of cells grown on different media provides only suggestive evidence that these enzymes may be important in cellular metabolism. Experiment 6 was conducted in an attempt to obtain evidence regarding their specific metabolic roles.

Experimental

The procedure outlined by Seaman (1963) was followed. CFE of E. coli 64 grown on acetate were added to incubation mixtures containing sodium acetate-2-C^{14}, unlabeled acetate, ATP, CoA and cofactors plus an unlabeled 2 to 5 C intermediate. After incubation for 30 min at 30 C under nitrogen, the protein was precipitated and the supernatant was passed through an ion exchange column. The distribution of radioactivity in the effluent was determined by paper chromatography followed by radioautography. Details are given in Appendix p. 18.

Results

In the presence of added unlabeled oxaloacetate, labeling was over 100 times greater in citrate than in malate (Table 13). This suggests the presence of the citrate condensing enzyme, citrate oxaloacetate-lyase (CoA acetylating) EC 4.1.3.7, which catalyzes the reaction,

$$\text{acetyl-CoA} + H_2O + \text{oxaloacetate} \rightleftharpoons \text{citrate} + \text{CoA}.$$

The fact that when glyoxylate was the added intermediate, malate was 58 times more heavily labeled than citrate, suggests that malate was formed from glyoxylate and acetyl-CoA through the action of malate synthase. The possibility that this could be due simply to an isotopic exchange reaction may be excluded because only slight incorporation into malate occurred in the presence of added citrate, α-ketoglutarate or pyruvate.

High radioactivity in malate (42,402 count/min) was also observed in the presence of unlabeled isocitrate, indicating that both isocitrate lyase and malate synthase were active. While radioactivity in malate in the presence of isocitrate was high, it was less than 1/3 of that observed when glyoxylate was added.

Figures 11, 12 and 13 are radioautograms of the acidic fractions obtained from incubation mixtures to which labeled acetate plus unlabeled oxaloacetate, isocitrate or glyoxylate was added.

Discussion

These results confirm the presence of both enzymes of the glyoxylate cycle at levels sufficient to be of importance to cells during growth on acetate. It seems probable that the following sequence of reactions is involved in the growth of E. coli 64 on acetate as the sole source of carbon.

$$\text{Acetate} + \text{ATP} \xrightleftharpoons{\text{Acetate kinase}} \text{Acetyl phosphate} + \text{ADP}$$

$$\text{Acetyl phosphate} + \text{CoA} \xrightleftharpoons{\text{Phosphate acetyltransferase}} \text{Acetyl-CoA} + \text{Pi}$$

$$\text{Acetyl-CoA} + \text{Oxaloacetate} + H_2O \xrightleftharpoons{\text{condensing enzyme}} \text{Citrate} + \text{CoA}$$

$$\text{Citrate} \xrightleftharpoons{\pm H_2O\ \text{Aconitase}} \underline{\text{Cis}}\text{-aconitate} \xrightleftharpoons{\pm H_2O} \text{Isocitrate}$$

$$\text{Isocitrate} \xrightleftharpoons{\text{Isocitrate lyase}} \text{Glyoxylate} + \text{Succinate}$$

$$\text{Glyoxylate} + \text{Acetyl-CoA} \xrightarrow{\text{Malate synthase}} \text{Malate} + \text{CoA}$$

Table 13. Distribution of radioactivity in citrate, malate and succinate plus fumarate after incubation of CFE of E. coli 64 in the presence of sodium acetate-2-C^{14} and single unlabeled intermediates.

Unlabeled intermediate added	Citrate count/min	Malate count/min	Succinate plus fumarate count/min
Oxaloacetate	168,472	1,438	1,448
Glyoxylate	1,964	114,616	17,182
Isocitrate	4,870	42,402	6,128
Citrate	1,223	6,012	822
Malate	716	278	559
α-ketoglutarate	593	738	1,018
Pyruvate	366	482	155
None	168	275	105

Fig. 11. Radioautogram showing the distribution of radioactivity in organic acids after incubation of CFE of E. coli 64 in the presence of sodium acetate-2-C^{14} and unlabeled oxaloacetate.

Fig. 12. Radioautogram showing the distribution of radioactivity in organic acids after incubation of CFE of E. coli 64 in the presence of sodium acetate-2-C^{14} and unlabeled isocitrate.

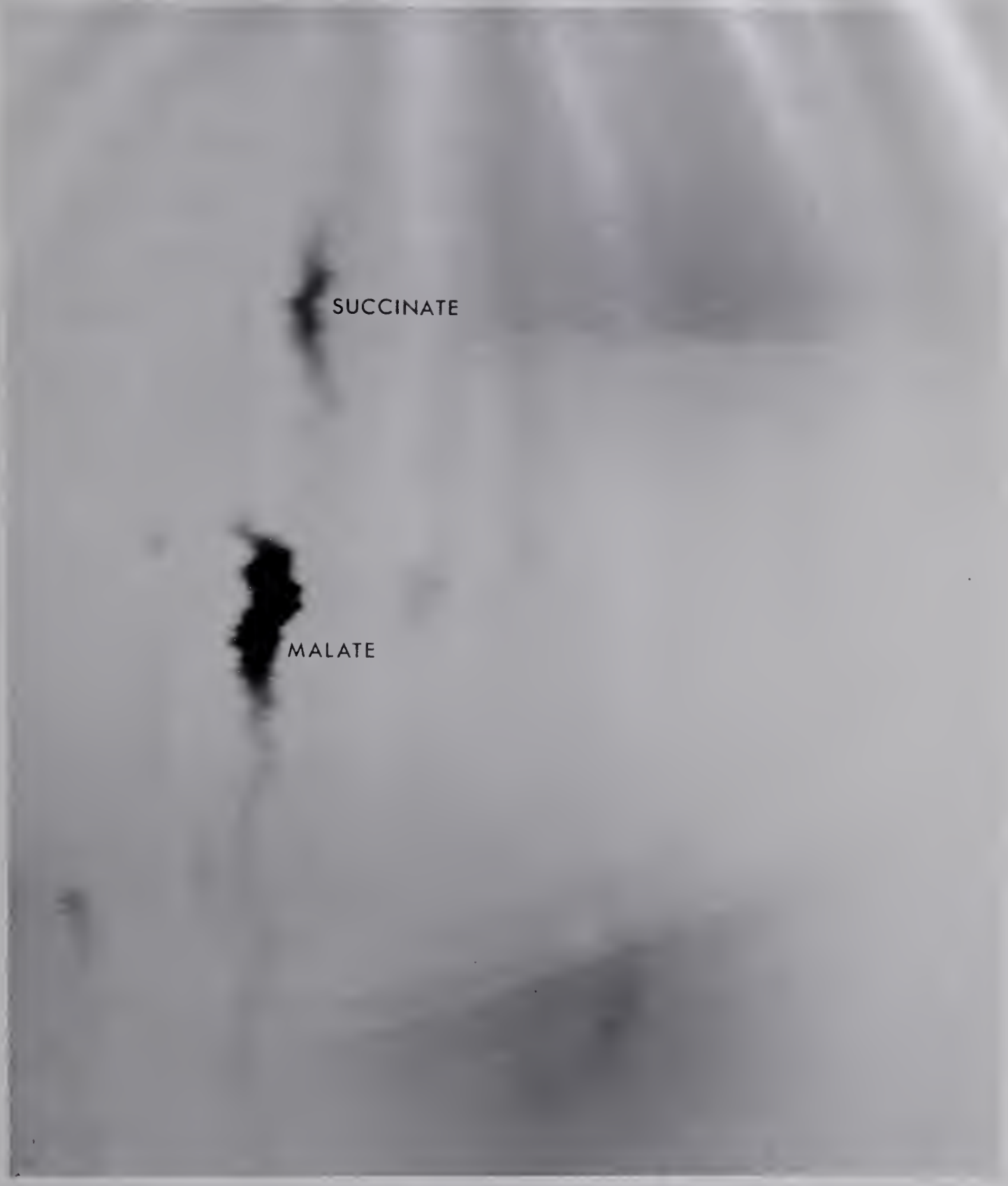


Fig. 13. Radioautogram showing the distribution of radioactivity in organic acids after incubation of CFE of E. coli 64 in the presence of sodium acetate-2-C^{14} and unlabeled glyoxylate.

Summary

1. When unlabeled oxaloacetate was added to an incubation mixture containing labeled acetate, ATP, CoA and cell-free extract of *E. coli* 64, there was heavy labeling in citrate, indicating the presence of citrate-condensing enzyme.
2. The presence of isocitrate lyase and malate synthase in the extract was confirmed by high activity in malate when glyoxylate or isocitrate was added to the incubation mixture.
3. A possible sequence of reactions by which *E. coli* 64 may use acetate as the sole source of carbon is listed.

GENERAL DISCUSSION

It is interesting to speculate regarding special functions that facultative anaerobes such as E. coli 64 could have in fermentation processes in the rumen.

Fermentation in the rumen is basically anaerobic, but appreciable amounts of oxygen are introduced into this organ with ingested feed and during rumination. Thus one function of facultative anaerobes could be the maintenance of anaerobiosis through utilization of ingested oxygen.

It is well established that acetate is the chief end-product of microbial digestion of carbohydrate in the rumen. Kornberg (1961) has stated that "organisms capable of forming specific enzymes are capable of so doing under a wide variety of environmental conditions, but may not fully express this genetic capability except when required for the survival of the organisms." E. coli 64 did not grow on acetate under strict anaerobic conditions, but under aerobic conditions it produced the enzymes of the glyoxylate cycle and grew readily on acetate as the sole source of carbon. If one assumes that E. coli 64 is a normal inhabitant of the rumen, the fact that it has retained the genes necessary for the synthesis of these enzymes is at least suggestive evidence that the glyoxylate cycle is functional in this organism at some stage of rumen fermentation. The ability of the organism to use acetate via the glyoxylate cycle could be particularly important after the readily available soluble carbohydrates are utilized and the major energy source for non-cellulolytic organisms becomes volatile fatty acids. Although the glyoxylate cycle has been mainly demonstrated in aerobic organisms, it has been found that the relative importance of this cycle increases under conditions of very low oxygen tension (Hogg and Kornberg, 1963; Levy and Scherbaum, 1965; Krebs and Lowenstein, 1960).

In the glyoxylate cycle one molecule of succinate and one pair of hydrogen atoms are formed from two molecules of acetate, whereas in the Krebs cycle one molecule of acetate yields two molecules of carbon dioxide and four pairs of hydrogen atoms. To the extent that the glyoxylate cycle may be operative in the rumen it would reduce the loss of energy as CO_2 from acetate and at the same time furnish 4-carbon intermediates to replace those drained from the Krebs cycle for synthetic purposes. In addition, the decreased requirement for hydrogen acceptors during the production of 4-carbon intermediates by the glyoxylate cycle rather than the Krebs cycle could be advantageous to the organism when growing on acetate under a low oxygen tension.

It seems possible that E. coli 64 and metabolically related organisms may have a role in lactate metabolism in the rumen. Lactate is present in the rumen in appreciable quantities when rations rich in carbohydrate are fed. If excessive amounts of lactic acid accumulate, the pH of the rumen fluid may become so low as to seriously interfere with overall microbial metabolism. Lactacidemia may result when a ration is suddenly altered to provide large amounts of readily fermentable carbohydrates, but if the change is gradual, high levels of carbohydrate are usually well tolerated because a gradual change provides time for the proliferation of organisms which ferment lactate. As has been indicated under Experiment 1, no marked increase in the abundance of coliforms relative to other organisms was observed in rumen fluid after the inclusion of a moderate amount of grain in the ration. None the less, it may be significant that, as was demonstrated in Experiment 2, E. coli 64 is capable of utilizing lactate either aerobically or anaerobically.

The facts that coliform organisms can utilize acetate and lactate, synthesize vitamins of the B-complex (Haenel and Müller-Beuthow, 1956) and use simple nitrogenous compounds as the sole source of nitrogen (Warner, 1956), suggest not only that they are well adapted to thrive in the rumen, but that they may also contribute pathways of metabolism that are essential to balanced or normal operation of the total rumen fermentation process.

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A P P E N D I X

Microbiological Methods

Viable count

The surface viable count of coliform organisms in the rumen was carried out by the method of Cruickshank (1960). MacConkey agar plates were prepared by dissolving 50 g of dehydrated MacConkey agar (Difco) in 1000 ml of distilled water and sterilizing in an autoclave at 15 lb. for 15 min. The sterile medium (pH 6.9) was aseptically poured in sterile Petri dishes. One ml of the thoroughly mixed rumen liquor was serially diluted to 10^{-8} with sterile 0.85% saline. After making up each dilution, the tubes were well shaken to ensure uniform mixing. From each dilution, 0.1 ml was poured onto the surface of the MacConkey agar plates and spread over the surface uniformly. Three replicates were prepared for each dilution and the plates were incubated at 37 C for 24 hr. The number of pink colonies on the plates was taken as representing the viable count of coliforms. Only those colonies more than 2 mm in diameter were counted.

In a few cases pour plates were prepared by inoculating 1 ml of each dilution of rumen fluid into about 12 ml of MacConkey agar which was previously melted and maintained at 45 C in a water bath. After thorough mixing the medium was allowed to set and the plates were incubated as before.

Total microscopic count

To count the total number of bacteria in the rumen fluid the method of Cruickshank (1960) was followed. The bacterial suspension was fixed by adding 2 or 3 drops of 40% formaldehyde per 100 ml of rumen fluid.

Freshly filtered methylene blue was added to a concentration of 0.1% to stain the bacteria. A drop of the appropriate dilution was then placed on the counting chamber of a haemocytometer (0.1 mm deep) and the bacteria were counted with an ordinary microscope using the 1/6 inch objective.

Indole test

The organism was grown in peptone water at 37 C and, after 2 days of incubation, 2 ml of the culture were taken in a test tube and an equal quantity of Ehrlich rosindole reagent (Cruickshank, 1960) was added. A rose pink color indicated a positive result.

Methyl red and Voges-Proskauer reactions

The organism was grown in a glucose phosphate medium as described by Cruickshank (1960). After 3 days of incubation at 37 C, one drop of a methyl red solution (0.1 g of methyl red dissolved in 300 ml of alcohol and made up to 500 ml with distilled water) was added to 5 ml of culture. A red color denoted a positive reaction. To 0.5 ml of the same culture 1 ml of 10% potassium hydroxide was added. Development of salmon color indicated the formation of acetoin and a positive V.P. reaction.

Eijkman's test

After growing the organism in MacConkey's fluid medium at 37 C, subcultures were made in the same medium and incubated at 44 C for 24 hr in a water bath. A positive Eijkman's test was indicated by gas production and turbidity.

Citrate utilization

This test determined the ability of the organism to grow on Koser's citrate medium consisting of 1.5 g sodium ammonium hydrogen phosphate, 1.0 g potassium dihydrogen phosphate, 0.2 g magnesium sulfate and 2.0 g citrate in 1 liter of distilled water.

Nitrate reduction

The organism was grown in a nitrate broth for 5 days at 37 C; 2 ml of each of a sulfanilic acid reagent containing 8 g sulfanilic acid and 1000 ml of 5 N acetic acid, and an alpha-naphthylamine reagent consisting of 5 g alpha-naphthylamine and 1000 ml of 5 N acetic acid were then added. The development of a rose color indicated the reduction of nitrate to nitrite.

Sugar fermentation tests

The basal medium consisted of peptone water to which 0.5-1% of the fermentable substance was added. Acid production was detected by addition of two drops of Andrade's indicator after 36 hr of incubation (Cruickshank, 1960). The hydrolysis of starch was tested by addition of a few drops of Lugol's iodine. Production of gas was detected by introducing a Durham's fermentation tube in each culture tube. Production of H_2S, gelatin liquefaction, urea hydrolysis and growth on litmus milk were tested as described in the Manual of Microbiological Methods (1957).

Cultivation of E. coli 64

The organism was cultivated according to the method of Glasky and Rafelson (1959). From the stock culture, the organism was inoculated into 10 ml of acetate medium consisting of the following:

	g
NaCl	0.09
$(NH_4)_2SO_4$	0.09
$MgSO_4.7H_2O$	0.01
Yeast extract	0.02
Potassium acetate	1.5
Phosphate buffer (0.1 M, pH 7.0)	100 ml

After 18 hr of incubation at 37 C, this culture was used to inoculate 100 ml of fresh acetate medium in an Erlenmeyer flask. This was incubated

on a horizontal rotary shaker at 37 C. At the end of 18 hr, a 10 ml aliquot from this flask was used to inoculate 100 ml of fresh acetate medium.

After four such transfers the cells were harvested by centrifugation at 15,000 rpm for 20 min, resuspended in 25 ml of fresh acetate medium and incubated as before. After 5 hr of incubation, the cells were harvested by centrifugation, washed with saline and adjusted to an OD of 2.0 with phosphate buffer. After an additional incubation for 15 min the bacterial suspension was used for tracer studies.

Radiobiochemical Procedures

Administration of labeled acetate

The uptake of labeled acetate by the cells was studied in Warburg flasks at 37 C. The main chamber contained 1.4 ml of bacterial suspension, 0.40 ml of phosphate buffer (0.1 M, pH 7.0), and 0.1 ml of 10 μM unlabeled potassium acetate. To absorb the carbon dioxide produced, 0.20 ml of 40% potassium hydroxide was pipetted into the central well. One μc (0.1 ml) of sodium acetate-2-C^{14}* with a specific activity of 1 μc/0.414 μmole was placed in the side arm. After equilibration for a few minutes the radioactive substrate was tipped from the side arm. At the end of incubation the reaction was stopped by rapidly chilling the flasks in dry ice-acetone bath (Glasky and Rafelson, 1959). The cells were separated from the radioactive medium by centrifugation at 15,000 rpm for 20 min in a Servall refrigerated centrifuge at 0 C. After decanting the supernatant the cell mass was washed once with ice-cold saline and centrifuged again.

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*Merck Sharpe and Dohme of Canada Ltd., Montreal.

Cell fractionation procedure

The isolation of metabolites from the washed cells was carried out by the methods described by Roberts et al. (1955) with slight modification. Three ml of hot absolute ethanol were added to the washed cells and, after keeping on a water bath at 50 C for 15 min, the contents were centrifuged and the supernatant saved. The extraction was repeated successively with hot 80% and 50% ethanol and finally with boiling distilled water.

The precipitate was suspended in a solution containing 2 ml of ether and 2 ml of 75% ethanol. After keeping in a water bath at 50 C for 15 min the suspension was centrifuged. The supernatants were combined and treated as described later.

The residue was treated with 6 ml of hot 5% trichloracetic acid, kept on a boiling water bath for 30 min, and centrifuged after cooling. The supernatant constituted the nucleic acid fraction.

The remaining precipitate was suspended in 6 N HCl and hydrolyzed at 110 C for 24 hr on a reflux condenser. The flasks containing the protein hydrolysates were evaporated under vacuum, the residue dissolved in a small volume of distilled water and an aliquot removed for radioactive determination. The rest was used for paper chromatography or further fractionated into acidic and basic amino acids by ion exchange chromatography.

The pooled supernatants were dried in vacuum on a water bath at 40 C. The dry residue was then treated successively with dry ethyl ether and distilled water to yield the lipid and water soluble fractions respectively. The aqueous portion was further fractionated into acidic (mainly organic acids), basic (mainly amino acids) and neutral (mainly sugars) fractions by the use of ion exchange resins as described by Canvin and Beevers (1961).

Chromatographic procedures

The following chromatographic procedures were used:

(a) Ion exchange chromatography

Preliminary treatment of the ion exchange resins consisted of suspending them in 5 times the quantity of deionized water, stirring with a glass rod and allowing to settle for 2-3 min. The lighter particles were decanted and the process repeated until particles of uniform size were obtained.

The cation exchange resin, Dowex 50W-X8 (H^+ form, 200-400 mesh)*, was poured into 6 x 1 cm columns supported by glass wool at the bottom and packed under a pressure of 5 lb. psi. About 60-100 ml of 2 N HCl were passed through the resin, followed by deionized water until the effluent was neutral.

The anion exchange resin, Dowex 1-X10 (Cl^- form, 200-400 mesh)*, was poured into similar columns. The chloride form of the resin was converted to the formate form by passing 1 M sodium formate through the column until the effluent was free of chloride ions when tested with acidified dilute silver nitrate solution. Then 30 ml of 0.1 N formic acid were passed through and the resin was finally washed with deionized water until the effluent was neutral.

The aqueous extract was first passed through the hydrogen column where the basic substances were adsorbed. The effluent, which contains the acidic and neutral substances, was passed through the formate column which adsorbs the acidic compounds. The effluent contains the neutral substances, mainly sugars. The acidic substances, mainly organic acids and phosphorylated compounds, were eluted from the formate column with 4 N formic acid. The basic substances, mainly amino acids, adsorbed by the hydrogen column, were eluted with 1 N ammonium hydroxide. These

*J. T. Baker Chemical Co., Phillipsburg, N.J.

three fractions were evaporated under vacuum at 40 C.

In a few cases, the protein hydrolysate was further fractionated into acidic and basic plus neutral amino acids by passing it through an acetate column. The acetate column was prepared in the same way as the formate column using Dowex 1-X10, except that 1 M sodium acetate and 30 ml of 0.1 N acetic acid were used. The acetate column adsorbs acidic amino acids and allows the neutral and basic ones to pass through (Hirs et al., 1954). The acidic amino acids were then eluted with 4 N acetic acid.

(b) Paper chromatography

Untreated Whatman No. 1 chromatographic paper was used. Chromatograms were run in a descending manner in a stainless steel chromatographic cabinet* without presaturation of the chamber with solvents. For two-dimensional chromatography of amino acids, the solvent systems used were n-butanol-acetic acid-water, 4:1:5 by volume, in the first direction and liquid phenol-water, 84:16 by volume in the second. Organic acids were separated using n-propanol-formic acid (sp gr 1.2, 98%)-water, 8:1:1 by volume (Schmidt et al., 1963) in the first direction and n-propanol-ammonia, 6:4 by volume (Isherwood and Hanes, 1953) in the second.

Organic acids were detected by spraying the paper with a mixture of aniline, xylose and methanol, 1:1:100 v/w/v, followed by heating at 90 C until brown spots developed. Amino acids were detected by spraying with 0.25% ninhydrin in acetone and heating.

Radioautography and radioassay

The radiobiochemical procedures used throughout this investigation are described below.

Radioactive spots were detected by exposing the chromatograms to Ilford "no screen" X-ray film** (35.6 x 43.2 cm) for 12-15 days. The

*Research Specialties Co., California.
**Picker X-Ray Engineering Ltd., Edmonton.

films were then developed and, by superimposing them on the chromatograms, the radioactive areas were marked with pencil. The identification of compounds was further confirmed by single-dimensional chromatography using at least two different solvents and standard markers. For this purpose 80% phenol (Aronoff, 1956), or a mixture of ether-formic acid-water, 5:2:1 by volume (Canvin and Beevers, 1961), was used for the identification of organic acids. The solvent systems, m-cresol saturated with 8.4 pH buffer (Aronoff, 1956) or acetone-n-butanol-water-diethylamine, 40:40:20:6 by volume, were used for single-dimensional chromatography of amino acids.

Radioactivity on single-dimensional chromatographic strips was detected by running them through an Actigraph III paper chromatogram scanner (Nuclear Chicago). Radioactive areas from papers were either cut, eluted and counted in a gas flow counter, or suspended in a scintillation solvent and counted in a liquid scintillation system as described below.

During the early part of the work radioactivities in different fractions were determined with an end-window gas flow counter (Nuclear Chicago)*. Aliquots of samples were pipetted onto aluminum planchets and counted at infinite thinness. Corrections were made for background. Under the conditions employed, the average counting efficiency was 16.7% so that 1 uc of sodium acetate-2-C^{14} gave approximately 370,000 counts per minute.

Carbon dioxide absorbed by potassium hydroxide in the central well of Warburg flasks was collected with repeated additions of carbonate-free water and precipitated by the addition of 1 ml of 10% barium chloride. The barium carbonate was filtered through glass fiber filter paper discs placed on a sintered glass disc on a vacuum filtration assembly. After washing with 95% ethanol, the filter paper discs were transferred carefully

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*Model D-47 Ser-2072. Courtesy of Dr. S. Zalik, Department of Plant Science, University of Alberta.

to aluminum planchets and counted at infinite thickness in the gas flow counter. Corrections were made only for background.

Radioactive determinations were also done using a temperature controlled liquid scintillation counting system (Nuclear Chicago Model 725) equipped with Auto/Subtract III for deducting background. The following settings gave a maximum counting efficiency for C^{14}:

	volts
L_2 discriminator control	2.0
L_3 " "	0.5
L_4 " "	1.5
L_5 " "	9.9
Data high voltage	980
Gate high voltage	1050

Scaler 1 at L_1 - L_2; Scaler 2 at L_3 - L_5
Channel 1 at L_1 ∞ ; Channel 2 at L_3 - L_5; Channel 3 at L_3 - L_4
Data attenuator at 1.0; temp 50 C

A series of quenched standards was prepared by addition of increasing amounts of chloroform to known quantities of radioactive material and after counting, the channel ratio, L_3 - L_4 (Scaler B) : L_3 - L_5 (Scaler A), was noted in each case. A quenching standard curve, with the percentage of efficiency of counting, count/min on Scaler A x 100 : dpm, on the ordinate and the channel ratio on the abscissa, was drawn for ready reference (Fig. 1). Under the conditions of counting employed, the counting efficiency was 77% for unquenched C^{14} samples and 1 μc yielded 1,600,000 count/min.

The scintillation solvent used was Polyether 611, which consisted of a 6:1:1 mixture by volume of dioxane*, anisole*, 1,2-dimethoxyethane* plus 12 g/liter of PPO** (2,5-diphenyloxazole) and 0.5 g/liter of POPOP** (p-Bis [2-(5-phenyloxazolyl)] -Benzene). This solution was transferred in 15 ml quantities to screw-capped bottles to which an aliquot of the material to be counted was added. After thorough mixing, each sample was counted for 40 min.

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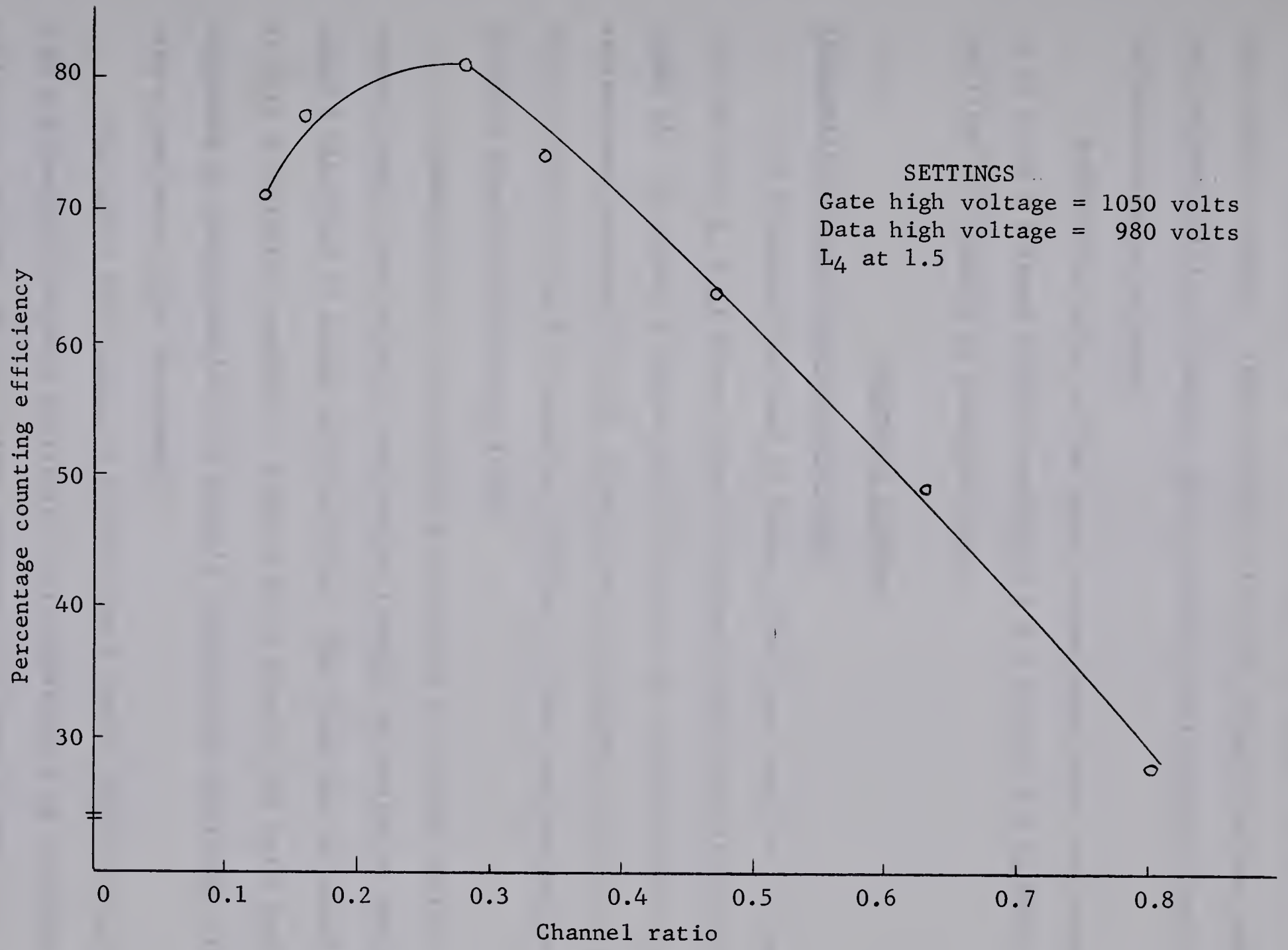
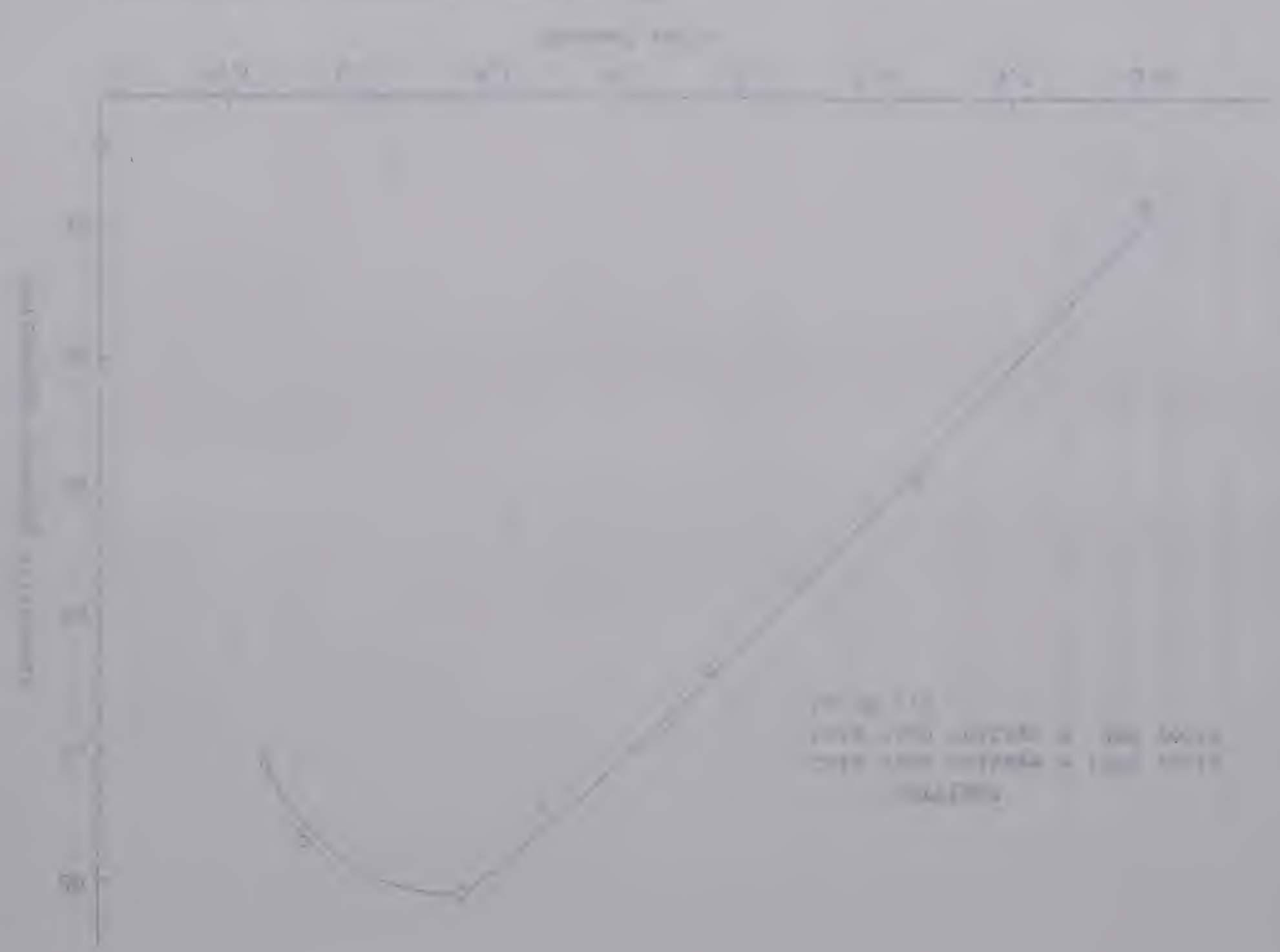
*Fisher Scientific Co. Ltd., Edmonton.
**Nuclear Chicago.



Appendix Fig. 1. Quenching curve for C^{14}.



Carbon dioxide trapped by potassium hydroxide in the central well of Warburg flasks was collected with small additions of carbonate-free distilled water. After suitable dilution, 50 µliters were pipetted into Polyether 611 and counted in the liquid scintillation system with the same settings as above.

Radioactive areas on the paper chromatograms were cut, suspended in 15 ml of a toluene solution containing 4.0 g PPO and 100 mg POPOP per liter and counted as described above.

Enzymatic Studies

Preparation of cell-free extracts (CFE)

The procedure outlined by Seaman (1963) was used for the preparation of CFE. *E. coli* 64 was grown in acetate medium described previously (page 3). To obtain a better yield of cells, the concentration of yeast extract was increased to 0.2%. After 4 serial transfers, 100 ml of a 24-hr culture was used to inoculate 5000 ml of fresh acetate medium in a 12-liter round-bottomed Pyrex flask.

Compressed air was sterilized by passing it through sterile cotton plugs, after which it was bubbled through the medium at approximately 3 liter/min to ensure good aeration. The flask was incubated at 37 C in a 'walk-in' incubator. After 8 hr of growth, the cells were harvested by centrifugation in a Servall refrigerated centrifuge fitted with a continuous flow attachment*.

The cells (wet weight, 2 g/liter) were washed thrice in ice-cold 0.05 M potassium phosphate buffer (pH 7.0) containing 2 µM of reduced glutathione (Sigma) per ml. After suspension in fresh buffer to a concentration of 30% (w/v), the cells were disrupted in a Raytheon 10-KC Sonic Oscillator* for 25 min with a current output of 1.2 amp and tap water as coolant.

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*Through the courtesy of Dr. G. E. Myers, Department of Microbiology, University of Alberta.

During subsequent procedures care was taken to ensure that the tubes containing the extracts were always kept immersed in ice lest the sensitive enzymes might be inactivated. The cell debris was removed by centrifugation at 15,000 rpm for 45 min at 0 C. The supernatant was dispensed in 1 ml quantities in screw-capped bottles and stored at -10 C until used.

Assay of acetate kinase

To determine which of the two acetate-activating systems was present in the extracts, the method of Seaman (1963) was followed. The extracts were first rendered low in CoA by treating 5 ml with 1.25 ml of Dowex 1-X4* (50-100 mesh, H^+ form). After 10 min the resin was separated by centrifuging in a table model centrifuge and the supernatant was withdrawn for use.

The substrate solution consisted of a mixture of 25 ml of 3.2 M potassium acetate, 0.5 ml of 1 N Tris-(hydroxymethyl)-aminomethane buffer (pH 7.4) and 1.0 ml of 1 M $MgCl_2.6H_2O$. Neutralized hydroxylamine solution was prepared by mixing equal volumes of 28% hydroxylamine hydrochloride and 4 N potassium hydroxide. Coenzyme A and ATP were obtained from Sigma Chemical Co., St. Louis, Mo.; 6.6 mg of CoA dissolved in 10 ml of distilled water yielded approximately 250 units/ml (Seaman, 1963). The ingredients except CFE were mixed as described below and incubated at 30 C on a water bath.

	Tube Numbers		
	1	2	3
Ingredient	ml	ml	ml
Substrate solution	0.30	0.30	0.30
Neutralized hydroxylamine	0.35	0.35	0.35
0.1 M ATP (Sigma)	0.10	0.10	0.10
1 M KF	0.05	0.05	0.05
0.2 M reduced glutathione (Sigma)	0.05	0.05	0.05
Distilled water	0.15	0.05	-
Coenzyme A (Sigma) 250 units/ml	-	-	0.05
Cell-free extract (1:10 dilution)	-	0.10	0.10

*J. T. Baker.

After equilibration for 5 min, CFE was added and after 20 min the incubation was stopped by the addition of 1.0 ml of 10% trichloracetic acid. Then 4.0 ml of 1.25% ferric chloride in 1 N HCl were added to complex the acetyl hydroxamate formed (Jones and Lipmann, 1955). The tubes were centrifuged and the intensity of the color of the supernatant was measured in a Beckman DU Spectrophotometer at 540 mμ.

Assay of phosphate acetyltransferase

This assay was based on the method described by Stadtman (1955). The following ingredients were pipetted into a centrifuge tube:

	ml
Tris buffer (0.1 M, pH 8.0)	0.10
Lithium acetyl phosphate (0.06 M) (Sigma)	0.10
CoA (Sigma) 50 units/ml	0.10
Cysteine hydrochloride (0.1 M)	0.10
Cell-free extract (Dowex-1 treated) (1:10)	0.20

Another tube, containing the same ingredients as above except that CFE was substituted by distilled water, served as a blank. The mixture was incubated at 28 C for 5 min; 0.1 ml of 0.5 M potassium arsenate was added and incubation continued for another 15 min. The residual acetyl phosphate at the end of incubation in both the tubes was determined by the addition of 1.0 ml of neutralized hydroxylamine solution. After 5 min, 1.0 ml of distilled water and 3.0 ml of a ferric chloride reagent prepared by mixing equal volumes of 5% $FeCl_3$ in 3 N HCl and 12% trichloracetic acid were added. After centrifugation, the supernatant was removed and the color intensity read at 540 mμ in a Beckman DU Spectrophotometer. A solution containing 2.7 ml of distilled water and 3.0 ml of ferric chloride reagent was used to zero the spectrophotometer.

Assay of the enzymes of the glyoxylate cycle

(a) Assay of isocitrate lyase

The reaction mixture consisted of the following:

	ml
Tris buffer (pH 7.2; 0.2 M)	0.50
$MgCl_2.6H_2O$ (2.860%)	0.50
Cysteine hydrochloride (0.1454%)	0.50
Phenylhydrazine hydrochloride (0.216%) (BDH)	0.50
CFE (acetate, 8 hr)	as indicated
Distilled water to a total of	3.0

The ingredients were pipetted into the cuvettes of a Beckman DU Spectrophotometer and the reaction was started by the addition of 5 μmoles of sodium isocitrate*. The change with time in the absorbancy at 324 mμ was noted.

(b) Reversibility of isocitrate lyase

The reaction mixture contained the following:

	ml
Tris buffer (0.2 M; pH 7.2)	0.50
$MgCl_2.6H_2O$ (2.860%)	0.50
Cysteine hydrochloride (0.1454%)	0.50
*$NADP^+$ (0.554 μmoles)	0.20
CFE (acetate, 8 hr)	0.05
Substrate	as indicated
Distilled water to a total of	3.0

To demonstrate isocitrate dehydrogenase, the reaction was started by the addition of 5 μmoles of isocitrate at zero time and the changes with time in the absorbancy at 340 mμ were observed. In the assay for the reversibility of isocitrate lyase, 20 μmoles of glyoxylate and 10 μmoles of succinate were added at zero time.

(c) Specific activity of isocitrate lyase

The following ingredients were pipetted into conical centrifuge tubes:

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*Sigma

	ml
Tris buffer (0.2 M; pH 7.2)	1.00
$MgCl_2.6H_2O$ (2.860%)	0.50
Cysteine hydrochloride (0.1454%)	0.50
Isocitrate (5 μmoles)	0.20
Distilled water	0.70
CFE after appropriate growth	0.10
Total	3.00

The tubes were incubated for 10 min at 30 C in a water bath. The head space was filled with nitrogen and covered with a marble. At the end of incubation, 0.2 ml of 80% trichloracetic acid was added and the tubes were centrifuged at 2800 rpm for 10 min in a table model centrifuge. The concentration of the glyoxylate formed in the supernatant was then determined.

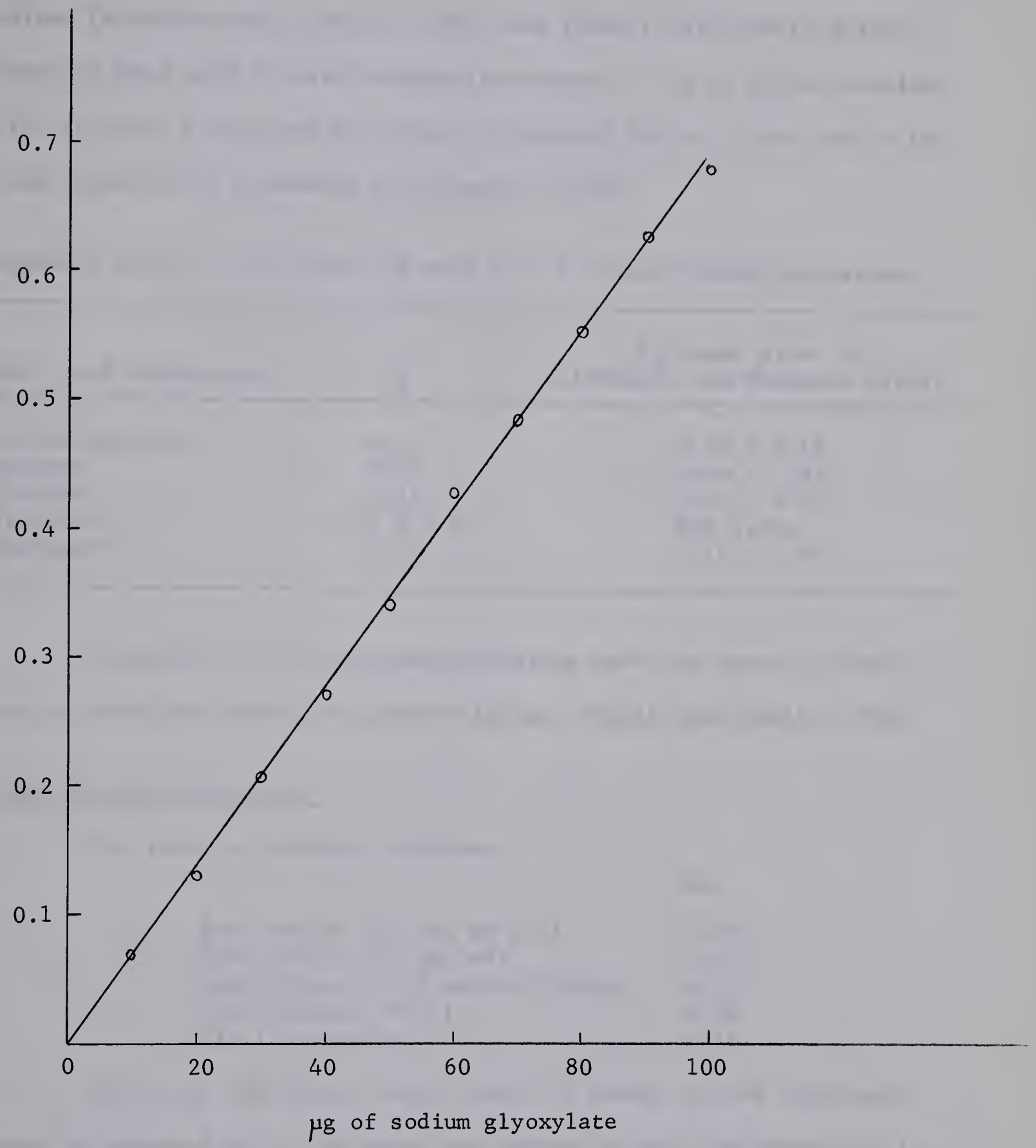
(d) Estimation of glyoxylate

To graded concentrations of standard sodium glyoxylate solutions, 2.0 ml of 0.05% 2,4-dinitrophenylhydrazine* (2,4-DNP) in 2 N HCl were added, followed by the addition of distilled water to a total of 5.0 ml. The tubes were mixed well and allowed to remain for 20 min at room temperature to facilitate the quantitative conversion of all the glyoxylate to its 2,4-DNP derivative. Then 4.0 ml of 1.5 N sodium hydroxide were added and the color intensity was read within 3 min in a Beckman DU Spectrophotometer at 540 mμ. From a standard curve so obtained (Fig. 2), the quantity of glyoxylate in the protein-free supernatant of the reaction mixture was determined.

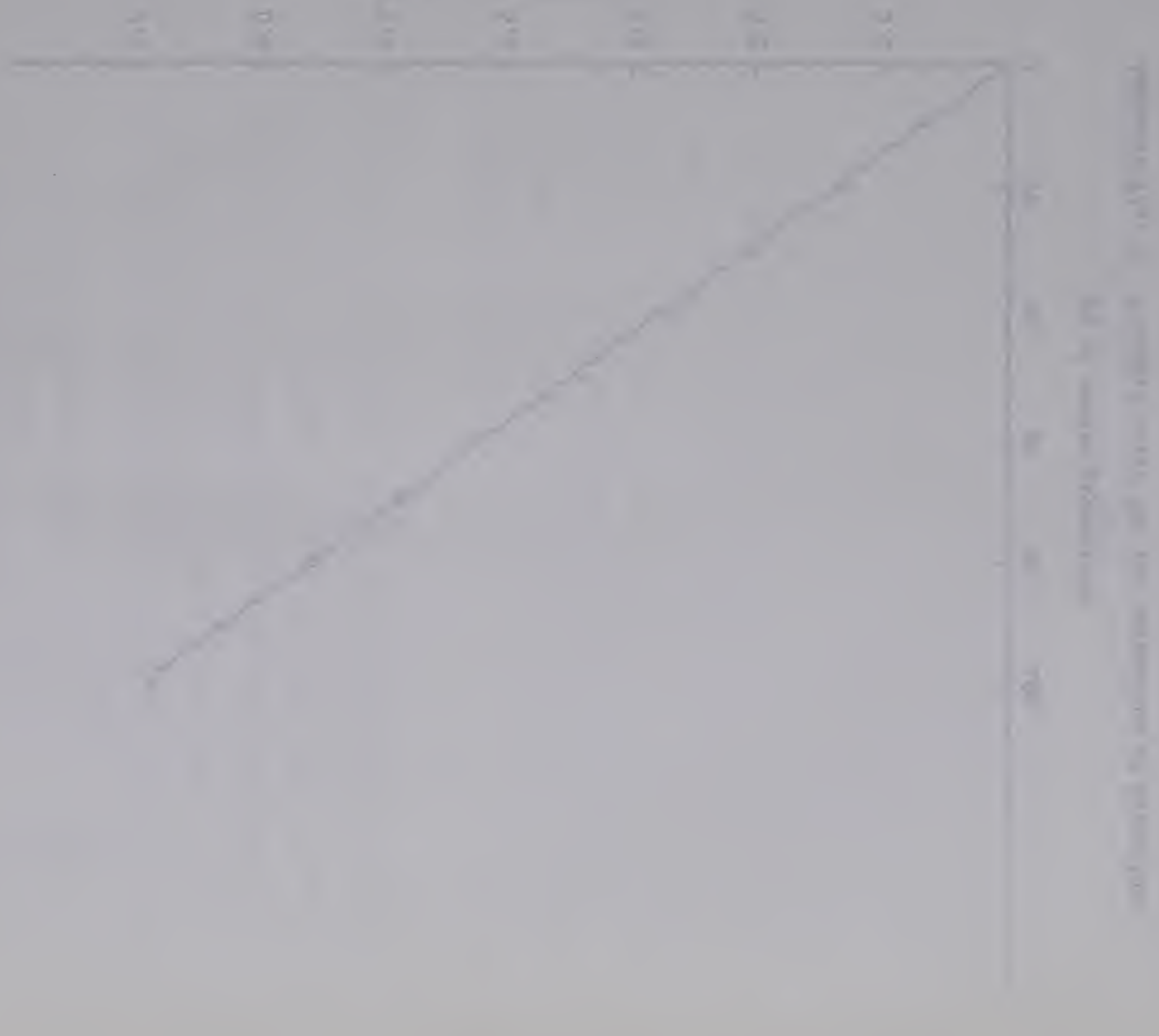
(e) Paper chromatographic identification of glyoxylic 2,4-dinitrophenylhydrazone

The presence of glyoxylic 2,4-dinitrophenylhydrazone in the reaction mixture was confirmed by extraction with ethyl acetate, spotting on Whatman No. 1 paper and running a descending chromatogram along with markers. After

*Fisher Scientific Co. Ltd.



Appendix Fig. 2. Standard curve for the estimation of glyoxylate



trying several solvent systems, in most of which there was long streaking on the spots, a mixture of n-butanol-ethanol-0.5 N ammonia, 7:1:2 by volume (el-Hawary and Thompson, 1953) was found to yield well defined spots of keto acid 2,4-dinitrophenylhydrazones. The R_f values obtained with standard hydrazones are given in Appendix Table 1; they are in the range reported by el-Hawary and Thompson (1953).

Appendix Table 1. R_f values of keto acid 2,4-dinitrophenylhydrazones

Keto acid derivative	R_f	R_f range given by el-Hawary and Thompson (1953)
α-ketoglutaric	0.11	0.08 - 0.15
Acetone	0.87	0.90 - 0.95
Pyruvic	0.33	0.35 - 0.47
Glyoxylic	0.25 & 0.37	Not given
Oxaloacetic	0.21	0.19 - 0.29

Glyoxylic 2,4-dinitrophenylhydrazone gave two spots, probably due to isomerism around the carbon-nitrogen double bond (Smith, 1960).

Assay of malate synthase

The reaction mixture contained:

	ml
Tris buffer (0.1 M; pH 8.0)	0.50
$MgCl_2.6H_2O$ (15 µmoles)	0.07
Acetyl-CoA (0.25 µmoles)(Sigma)	0.05
CFE (acetate, 8 hr)	0.05
Distilled water	2.33

After the ingredients were mixed, no change in the absorbancy could be observed which indicated the absence of acyl-CoA deacylase in the extracts. The reaction was started by the addition of 2 µmoles of sodium glyoxylate monohydrate (Sigma) and the decrease in absorbancy at 232 mµ was noted against time in a Beckman DU Spectrophotometer.

Isotope studies on the enzymes of the glyoxylate cycle

The procedure of Seaman (1963) was followed. Incubations were done in single-armed Warburg flasks at 30 C under nitrogen. The reaction mixture consisted of the following:

	ml
Potassium phosphate buffer (0.1 M; pH 7.0)	0.10
Reduced glutathione (0.2 M) (Sigma)	0.05
CoA (Sigma) (250 units/ml)	0.10
$MgCl_2.6H_2O$ (0.1 M)	0.10
ATP (0.1 M) (Sigma)	0.10
Potassium acetate (0.03 M)	0.10
Sodium acetate-2-C^{14} (1 μc) (specific activity 1 μc/0.414 μmole)	0.10
Unlabeled substrate (10 μmoles each)	0.10
CFE (acetate, 8 hr)	0.25

One flask containing 0.1 ml of distilled water instead of the substrate solution served as the blank. The reaction was started by the addition of enzyme solution from the side-arm.

After 30 min of incubation, 3 ml of hot ethanol were added to each flask and kept on a water bath at 50 C for 20 min. The extraction process was repeated successively with 50% ethanol and boiling water. The combined supernatant was evaporated under vacuum at 40 C, dissolved in water and passed through a column of Dowex 50 W-X8 (hydrogen form). The acidic fraction was evaporated under vacuum, dissolved in a small volume of distilled water and spotted on Whatman No. 1 paper. A two-dimensional chromatogram was run using n-propanol-formic acid-water, 8:1:1 by volume as the first solvent and n-propanol-ammonia, 6:4 by volume as the second.

Using the radioautographic procedure already described, the active areas on the chromatogram were cut and counted in the liquid scintillation system by suspending them in the toluene solution (appendix page 11). The identification of the radioactive compounds was further confirmed by cochromatography.

Appendix Table 2
Viable coliform counts in rumen fluid from cows fed alfalfa hay

Cow	Lyssa				Calla			
Dilution of rumen fluid	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Date	MacConkey agar, no. colonies/plate							
Dec. 1/64								
Replicate I	35	26	14	4	32	20	8	5
" II	29	20	10	2	30	22	10	-
" III	29	21	12	3	30	19	9	7
Avg	31	22	12	3	31	20	9	4
Pour plate colonies			8			5		
Jan. 15/65								
Replicate I	30	22	15	4	20	15	10	4
" II	34	19	10	2	21	13	9	3
" III	30	20	8	-	25	14	11	4
Avg	31	20	11	2	22	14	10	4
Pour plate colonies		6				5		
Mar. 3/65								
Replicate I	10	4	-	-	12	8	1	-
" II	10	5	-	-	11	4	-	-
" III	13	6	-	-	13	6	3	-
Avg	11	5	-	-	12	6	1.3	-
Pour plate colonies		3 in 10^{-3} dilution			2			
July 1/65								
Replicate I	30	16	11	1	1	-	-	-
" II	28	22	7	1	-	-	-	-
" III	28	16	6	-	-	-	-	-
Avg	29	18	8	-	-	-	-	-
Pour plate colonies		6			4 in 10^{-3} dilution			

Appendix Table 3
Recovery and distribution of radioactivity after incubation of *E. coli* 64 for 1 hr with sodium acetate-2-C^{14}

Fraction	Activity (count/min)		
	Flask 1	Flask 2	Flask 3
CO_2 (as $BaCO_3$)	124,973	118,973	124,973
Incubation mixture	214,500	201,480	236,120
Total	339,473	320,453	361,093
Total C^{14} added	370,000	370,000	370,000
% recovered	91.8	86.6	97.6
Incubation mixture fractions:			
Incubation supernatant	56,416	38,418	69,782
Protein hydrolysate	111,120	114,880	117,480
Hot trichloracetic acid soluble fraction (nucleic acids)	12,132	12,546	10,980
Ether extracts (lipids)	9,511	10,824	9,275
Total aqueous fraction	20,140	21,734	24,000
Organic acids	14,800	13,280	17,160
Amino acids	2,181	4,482	3,896
Sugars	-	-	-
Total	209,319	198,402	231,517
% recovered from incubation mixture	97.6	98.5	98.1